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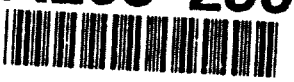

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One series of experiments has studied regeneration of the cut central processes of adult dorsal root ganglion cells into transplants of embryonic CNS tissue. The cut dorsal roots regenerate into spinal cord transplants and contain peptides characteristic of normal dorsal roots. Synapses are established that resemble those found in the normal dorsal horn, are functional, and appear to be permanent. Cut dorsal roots also regenerate into transplants of embryonic brain, but growth into spinal cord transplants is more robust. Another series of studies has used immunocytochemical, in situ hybridization, and cell counting methods to show that cutting the peripheral processes of dorsal root ganglion neurons has profound effects on tachykinin synthesis and can cause neuron death, whereas the neurons survive axotomy of their central processes and tachykinin synthesis is unaffected. The most recent studies have shown that embryonic transplants can rescue the axotomized neurons of Clarke's nucleus that would otherwise die.

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TABLE OF CONTENTS

FINAL REPORT

(5) INTRODUCTION

Embryonic CNS transplants provide a powerful experimental approach to understanding the features of the neural environment that interact with damaged neurons to support or enhance regeneration. Neurons thought to be incapable of growth (1) can not only regenerate if supplied with a transplant of fetal CNS but also form connections that are functional by electrophysiological and behavioral criteria (reviewed in 3;21). Although the mechanisms by which transplants restore function remain to be clarified, they provide both an experimental approach to understanding repair mechanisms and a potential therapeutic strategy (8).

Intraspinal transplants of brain (5;6;18) and spinal cord (2;16;20;23) grow and integrate with the host. The extent to which these transplants support regeneration of adult host intraspinal axons, however, is uncertain. It is also unclear whether axons that regenerate into intraspinal transplants retain the ability to synthesize normal proteins. The central processes of dorsal root ganglion (DRG) neurons provide an attractive model system in which to study these questions. First, it is well known that the cut central processes of adult DRG neurons regenerate along the dorsal root, but fail to traverse the dorsal root entry zone and re-enter the spinal cord (4;19). Second, horseradish peroxidase (HRP) transport methods can be used to identify dorsal root axons unambiguously within spinal cord or transplants. Third, DRG axons can be classified into subsets based on the presence of neuropeptides such as calcitonin gene-related peptide (CGRP) (7), and immunocytochemical methods can be used to identify these axons and therefore to determine whether the peptides are present in transplants. Because of these advantages, we proposed to use the adult DRG-fetal transplant model to study several features of axon growth into transplants (Specific Aim 3). We first used HRP transport methods and CGRP immunocytochemistry to show that cut dorsal root axons regenerate into transplants of embryonic spinal cord and that some of these axons contain CGRP (23).

To determine the functional potential of these regenerated dorsal root axons, it is important to establish whether or not they make synaptic contacts with neurons within the transplants. We therefore used HRP tracing methods and immunocytochemical techniques to show that regenerated dorsal roots form synapses within transplants of embryonic spinal cord and that these synapses resemble those formed in the dorsal horn of normal spinal cord (14). We have also shown that these synapses are functional (14a). Since the environment within which regenerated axons form synapses may also contribute to their functional potential, we used stereological methods to examine in detail the composition of transplanted spinal cord and compared it to the structures found in normal dorsal horn (14). We have also studied the development of these transplants and the growth and maturation of the regenerating dorsal root axons (15a).

It is unknown whether the properties of the transplants that allow regenerating axons to grow and establish synapses are specific to the normal target of the cut axons, or if they are common to embryonic CNS tissue generally. The early outgrowth of developing axons is thought to depend on signals that are expressed generally throughout the embryonic CNS, whereas growth within a target and synapse formation appear to rely on more specific cues (reviewed in 17). The requirements of regenerating axons both for early outgrowth (22;9) and for synapse formation (24) may differ from those of developing axons but have received relatively little attention. We therefore used quantitative morphological methods to investigate whether or not regenerating adult dorsal root axons can grow into and establish synapses in transplants of embryonic brain regions that are not their normal targets and whether the patterns of growth and synapse

formation differ in transplants of spinal cord and brain. In this way we distinguish characteristics of growth that are target-specific from those that are shared by non-target embryonic CNS tissue (15). We also showed a difference in the ability of CGRP-containing dorsal roots to regenerate into transplants of ventral or dorsal half of spinal cord (15b).

If the therapeutic potential of transplants is to be realized, it is important to understand the factors that contribute to the survival of transplanted neurons. DRG transplants provide advantages for understanding this problem, and Specific Aims 1 and 2 proposed to use DRG transplants to study factors that contribute to the survival of transplanted neurons and axon growth. We have carried out several studies that are related to these Aims. First, we compared the effect on survival of cutting the central or peripheral processes of newborn DRG neurons and also compared DRG neuron survival after cutting the sciatic nerve in adult or newborn rats (13). Second, we used immunocytochemical and histochemical methods to determine whether or not specific populations of DRG neurons are particularly likely to die after axotomy (12). Third, we used immunocytochemistry and in situ histochemistry to examine in detail the metabolic response to axotomy of tachykinin-containing DRG neurons (10;11). We have also studied the factors required for survival of axotomized neurons in Clarke's nucleus (11a).

(6) **BODY**

a. **Methods**

1. Surgical Methods. Female Sprague-Dawley rats (200-300g) are deeply anesthetized and undergo a laminectomy to expose the fourth lumbar (L4) segment. The adjacent dorsal roots are cut near the dorsal root entry zone and reflected caudally. A hemisection cavity 3-4mm in length is aspirated from the lumbar enlargement, the appropriate transplant is introduced into the cavity, and the L4 or L5 dorsal root stump is juxtaposed to the transplant. The surgical wound is then closed in layers. The surgical techniques have been described in detail in publications from this laboratory, as have the techniques for neural graft preparation (14;23, included in Appendix). For sciatic nerve section, the right sciatic nerve of deeply anesthetized adult or newborn rats is sectioned in the mid-thigh (see 13).

2. Labeling Methods.

i. Dorsal root labeling. Dorsal roots entering the transplants are labeled with 10% HRP and 1% WGA-HRP (wheat-germ agglutinin-conjugated HRP) as described (14;23; see Appendix).

ii. Sciatic nerve labeling. The sciatic nerve ipsilateral to the transplant is labeled with an intraneural injection of 0.75% cholera toxin-conjugated HRP or 2% WGA-HRP (14).

iii. CGRP and tachykinin immunocytochemistry. Sections are prepared for LM and EM examination with the PAP technique (14).

iv. In situ hybridization. These methods are described in publications from this laboratory (9;10).

3. Stereological and morphometric analyses. These methods are detailed (14).

b. **Results**

Projects 6-9 are related to Specific Aims 1 and 2 of the Contract and Projects 1-5 to Specific Aim 3.

1. Adult dorsal root axons regenerate into transplants of embryonic spinal cord. *(Tessler, A., B.T. Himes, J. Houle, and P.J. Reier. 1988. Regeneration of adult dorsal root axons into transplants of embryonic spinal cord. J. Comp. Neurol. 270:537 548.)* Anatomical tracing methods that use transport or diffusion of HRP demonstrate that severed host dorsal roots grow into transplants of embryonic day 14 (E14) spinal cord. Most of these regenerated axons remain within 2mm of the interface with the host dorsal root, but some axons penetrate as far as 3mm. Many more regenerated axons are demonstrated with immunocytochemical methods that visualize CGRP. The results of this study therefore show that transplants of embryonic spinal cord support or enhance the regeneration of cut adult host DRG axons and that many of those that regenerate continue to synthesize peptides that they make normally.

2. Regenerated DRG axons form synapses in transplants of embryonic spinal cord. *(Itoh, Y. and A. Tessler. 1990. Ultrastructural organization of regenerated adult dorsal root axons within transplants of fetal spinal cord. J. Comp. Neurol. 292:396-411.)*

Labeling methods that use diffusion of WGA-HRP and CGRP immunocytochemistry show that regenerated DRG axons establish synaptic contacts with neurons in embryonic spinal cord transplants. As in normal dorsal horn, the majority of the CGRP-labeled axon terminals are axodendritic, but a large number are also axosomatic and axoaxonic. Regenerated dorsal root axon terminals in transplants are significantly larger than those found in normal dorsal horn, and their synaptic contact length is also increased, suggesting that a compensatory mechanism for increasing synaptic efficacy might occur within transplants. The results of this study show that regenerated dorsal root axons form synapses within transplants and that these synapses retain characteristics of those found in normal spinal cord. These results therefore encourage the expectation that transplants may one day be used to restore neural circuits damaged by trauma or disease.

3. Cut DRG axons regenerate into transplants of brain and form synapses there. *(Itoh, Y. and A. Tessler. 1990. Regeneration of adult dorsal root axons into transplants of spinal cord and brain: A comparison of growth and synapse formation in appropriate and inappropriate targets. J.Comp.Neurol. 302:272-293.*

We used immunocytochemical methods for labeling CGRP to examine whether cut dorsal root axons regenerate into and establish transplants in embryonic brain transplants and to compare the patterns of growth in brain and spinal cord transplants. Transplants of embryonic hippocampus, cerebellum, and neocortex and embryonic spinal cord were studied. CGRP-immunoreactive axons regenerate into all of the brain regions and form synapses in the neocortex and cerebellum transplants in which they were sought. Synapses are far rarer than we have observed in solid spinal cord transplants, and the patterns of ingrowth differ in transplants of brain and spinal cord. Both the area fraction and area density occupied by regenerated axons in spinal cord transplants are significantly larger than in neocortex or cerebellum transplants. In addition, the distribution of regenerated axons within spinal cord transplants is heterogeneous, since areas of either dense or sparse ingrowth are observed, whereas the distribution in transplants of brain is homogeneous but sparse. Several measurements of the extent of axon distribution, however, including area, longest axis, and length of lateral extension, indicate that CGRP-labeled axons spread more widely in neocortex transplants than in solid transplants of spinal cord or cerebellum. These results indicate that embryonic CNS tissues that are not normal targets support or enhance the growth of severed dorsal roots and suggest that the conditions that constitute a permissive environment for regenerating axons are relatively non-specific. Embryonic spinal cord, the normal target of dorsal roots, appears to supply additional more specific cues that enable regenerating axons to grow and arborize within the transplant and to establish relatively normal numbers of synapses. These cues appear to depend at least in part on the integrity of transplant structure, since growth into solid transplants of spinal cord exceeds growth into cell suspensions.

We have also found that dorsal roots regenerate into transplants of either ventral or dorsal spinal cord but that growth into dorsal spinal cord transplants is more robust. Since dorsal spinal cord is the normal target of most dorsal root axons, these results are consistent with the idea that regenerating axons remain sensitive to specific cues provided by their normal targets. *(Itoh, Y., Kowada, M., and Tessler, A. (1993) Regeneration of adult dorsal root axons into transplants of dorsal or ventral half of spinal cord. Adv. Stereotactic Functional Neurosurg. In Press.)*

4. Time course of dorsal root axon regeneration into transplants of fetal spinal cord. *(Itoh, Y., Sugawara, T., Kowada, M., and Tessler, A. (1992) Time course of dorsal root axon regeneration into transplants of fetal spinal cord: I. A light microscopic study. J.Comp.Neurol. 323:198-208; Itoh, Y., Sugawara, T., Kowada, M., and Tessler, A. (1993) Time course of dorsal root regeneration into transplants of fetal spinal cord: an electron microscopic study. Submitted for publication).*

The aims of this study were to describe the progression of dorsal root axon growth within transplants and to determine whether the regenerated axons persisted within the transplants or eventually withdrew. CGRP-immunoreactive dorsal root axons began to regenerate into transplants within 24 hours, formed dense bundles by 4 weeks, and were still present at 60 weeks, the longest survival period examined. Quantitative analysis showed that the area of the transplants occupied by CGRP-labeled axons and the distribution area of the labeled axons within the transplants increased until 12 weeks and persisted unchanged for over 48 weeks. Electron microscopy showed that the regenerated axons made synapses with dendrites and perikarya of transplant neurons by 1 week after axotomy and axoaxonic synapses by 4 weeks. The synaptic density of regenerated CGRP-labeled terminals increased for 24 weeks and then remained unchanged. These results indicate that regenerated dorsal root axons and their synaptic terminals are permanently maintained within transplants of fetal spinal cord and suggest that the transplants can contribute to the permanent restoration of damaged intraspinal neural circuits.

5. Regenerated dorsal root axons establish functional connections with neurons in fetal spinal cord transplants. *(Itoh, Y., Tessler, A., Kowada, M., and Pinter, M. Electrophysiological responses in fetal spinal cord transplants evoked by regenerated dorsal root axons Adv.Stereotact.Funct.Neurosurg. (In Press).*

Electrophysiological methods demonstrated the presence in transplants of synaptically-driven neuronal activity in response to electrical stimulation of regenerated roots. In some cases we observed monosynaptic EPSPs in transplant neurons following root stimulation using intracellular recording. Together with our observation that transplant neurons send axons into host sciatic nerve, these results suggest that transplants can act as relay stations that can contribute to at least partial restoration of injured circuits.

6. Transplant-mediated rescue of Clarke's nucleus (CN) neurons. *(Himes, B.T., Goldberger, M.E., and Tessler, A. (1993) Grafts of fetal CNS tissue rescue axotomized Clarke's nucleus neurons in adult and neonatal operates (submitted for publication).*

We have used the neurons of CN to study issues related to survival after injury. Injury at the T8 segment in newborn rats causes 40% of the ipsilateral CN neurons at L1 to die, and the same lesion in adults kills 30% of the neurons. Transplants of E14 spinal cord, cerebellum, and neocortex enable almost all of the axotomized neurons to survive for at least 2-3 months in both adult and newborn animals. Because all 3 of these tissues contain high levels of Neurotrophin-3 mRNA (NT-3), NT-3 may be the factor responsible for the rescue. The results of our recent transplant studies support this idea: axotomized CN neurons are salvaged by kidney, which synthesizes large quantities of NT-3 mRNA (Maisonpierre et al., '90;Ernfors et al., '90) but not by embryonic striatum, which expresses very little if any (Maisonpierre et al., '90).

7. DRG neurons die after sciatic nerve section. *(Himes, B.T. and A. Tessler (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. J. Comp. Neurol. 284:215-230.*

We used immunohistochemical and histochemical methods along with cell counting techniques to demonstrate that DRG neurons die after sectioning their peripheral processes and to show that several subsets of DRG neurons are equally likely to die after this injury. DRG neurons do not die after section of their central process, suggesting that death is the result of causes other than axotomy alone.

8. Changes in tachykinin synthesis after axotomy. *(Henken, D.B., A. Tessler, et al. (1988) In situ hybridization of mRNA for beta-preprotachykinin and presomatostatin in adult rat dorsal root ganglia: comparison with immunocytochemical localization. J. Neurocytol. 17:671-681; Henken, D.B., Battisti, W., Chesselet, M-F., Murray, M., and A. Tessler (1990) Expression of beta-preprotachykinins in rat dorsal root ganglion cells following peripheral or central axotomy. Neurosci.29:733-742.*

We used in situ hybridization and immunocytochemical methods to show that peripheral axotomy but not central axotomy causes reduced synthesis of the mRNAs that encode tachykinins and the tachykinins themselves. Changes in synthesis therefore are due to mechanisms other than a nonspecific response to injury, such as reduced levels of Nerve Growth Factor (NGF) which is derived from the peripheral but not the central target of DRG neurons.

9. Changes in levels of substance P (SP) and CGRP in DRG and dorsal horn after neonatal sciatic nerve lesion. *(One abstract has been published with F.Nothias and M.Murray, one in press in Restor.Neurol.Neurosci. A manuscript is in preparation).*

We had previously found that sciatic nerve section in newborn rats killed 50% of L5 DRG neurons, but that the survivors could restore dorsal horn SP levels to normal (Himes and Tessler, '89). Here we used immunocytochemistry, in situ hybridization histochemistry, and radioimmunoassay to determine the mechanism responsible. We excluded both selective survival of peptidergic neurons and increased constitutive levels of expression of peptide genes as mechanisms accounting for the recovery of dorsal horn SP and CGRP after neonatal sciatic nerve section. Experiments using additional lesions suggested that the recovery is due at least in part to sprouting from surviving L4 and L5 DRG neurons and neurons in DRG rostral and caudal to L4 and L5. Modifications in metabolism that increase peptide half-lives in surviving L4 and L5 DRG neurons may also contribute.

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(7) CONCLUSIONS

It follows from this work that cut dorsal roots of adult DRG neurons regenerate into transplants of embryonic spinal cord, whereas they do not regenerate into spinal cord in the absence of a transplant. The regenerated dorsal roots contain peptides found in normal dorsal roots, and they establish synapses that resemble those found in normal spinal cord and are functionally effective. These findings are encouraging for the hope that transplants may one day serve a therapeutic function and contribute to the restoration of damaged neural circuits.

The stimulus that elicits or supports dorsal root regeneration appears not to be specific to embryonic spinal cord, the normal target of dorsal roots, because dorsal roots also regenerate into transplants of embryonic brain. Regeneration into brain regions is less robust than into spinal cord, however, and synapses are far less frequent, suggesting that regenerating adult neurons require specific cues for growth within a target and synapse formation. Cutting their peripheral process produces profound metabolic changes in several subsets of DRG neurons and can cause the cells to die, whereas the neurons survive central axotomy and changes in tachykinin synthesis are not observed.

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(9) APPENDIX

Enclosed is a copy of each of the articles published with the support of this Contract.

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Ultrastructural Organization of Regenerated Adult Dorsal Root Axons Within Transplants of Fetal Spinal Cord

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ABSTRACT

It has previously been demonstrated that the severed central branches of adult mammalian dorsal root ganglion cells regenerate into transplants of fetal spinal cord. The aim of this study was to determine whether these regenerating axons form synapses, and, if they do, to characterize them morphologically.

Embryonic day 14 or 15 spinal cord was transplanted into the lumbar enlargement of adult Sprague-Dawley rats, and the L4 or L5 dorsal root was cut and then juxtaposed to the transplant. One to 3 months later the regenerated dorsal roots were labeled by anterograde filling with wheat germ agglutinin-horseradish peroxidase (WGA-HRP) or by immunocytochemistry for calcitonin gene-related peptide (CGRP).

Dorsal root labeling with WGA-HRP demonstrated that regenerated axon terminals made synaptic contacts within transplants, and stereological electron microscopic analysis demonstrated that CGRP-immunoreactive axon terminals occupied an average of 9% of the neuropil within 2 mm of the dorsal root-transplant interface. The majority of synapses were axodendritic, but a significant percentage were axosomatic or axoaxonic. Since axoaxonic synapses were observed in transplants in which both pre- and postsynaptic profiles of axoaxonic synapses were labeled for CGRP, some regenerated axons apparently form synapses with each other. Approximately 90% of synaptic contacts were simple, 9% were complex, and 25% of the complex terminals were immunopositive for CGRP. Glia occupied 25% of the neuropil within 1 mm of the dorsal root-transplant interface, but only 6% of the neuropil 1–2 mm from the interface.

We also performed a stereological analysis of the neuropil in lamina I. The area fractions of neuropil occupied by myelinated axons, perikarya, and dendrites were similar in transplants and in lamina I. However, the area fraction occupied by unmyelinated axons was significantly smaller in transplants, and the area fraction occupied by axon terminals was significantly larger in transplants compared with lamina I. Regenerated CGRP-immunoreactive synaptic terminals in transplants were significantly larger than in normal lamina I, and their synaptic contact length was also increased, suggesting that a compensatory mechanism for increasing synaptic efficiency might occur within the transplants. Synaptic density, however, was significantly reduced in transplants, indicating a smaller number of synaptic terminals per unit area. In lamina I, as in the transplant, most synapses were axodendritic, but the percentage of axosomatic and axoaxonic terminals was lower in lamina I than in the transplants. The area occupied by glia in lamina I was similar to

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that observed 1–2 mm from the dorsal root-transplant interface, but lower than that observed 0–1 mm from the interface.

The results of this study show that regenerated primary afferent axons form synapses within transplants. These synapses retain many of the characteristics of primary afferent synapses in normal dorsal horn, suggesting that transplants may provide a strategy to restore some of the properties of damaged neural circuits.

Key words: CNS regeneration, embryonic spinal cord transplant, WGA-HRP, anterograde transport, calcitonin gene-related peptide, immunocytochemistry, electron microscopy

The severed central branches of adult mammalian dorsal root ganglion (DRG) neurons regenerate along the dorsal root, but few, if any, penetrate the dorsal root entry zone and regrow into the spinal cord (Reier et al., '83a, '86; Liuzzi and Lasek, '87; Reier et al., '89). When transplants of fetal spinal cord are substituted for adult spinal cord, however, DRG axons regenerate into the transplants (Tessler et al., '88). In order to determine the functional potential of these regenerated axons, it is important to establish whether they make synaptic contacts with neurons in the transplant. Although synapses have been described within intraspinal transplants of embryonic spinal cord (Reier et al., '86), it is not known whether they derive from host or from transplanted neurons. To determine whether synapses within the transplant derived from regenerated DRG axons, in the present study we have therefore identified regenerated axons using wheat germ agglutinin-conjugated horseradish peroxidase (WGA-HRP) transport and immunocytochemical methods (Tessler et al., '88). We also determined the synaptic density and characterized the synaptic terminals.

The environment within which such regenerated axons make synaptic contacts may also be a significant factor in determining their functional potential. Transplants of embryonic spinal cord contain all the neural and glial elements that make up the neuropil of normal spinal cord (Reier et al., '86). However, the composition of transplanted spinal cord has not been studied in detail. In the present study, we performed stereological analyses to determine the area fraction occupied by myelinated and unmyelinated axons, glia, terminals, perikarya, and dendrites.

In addition to characterizing the transplant in terms of the formation of synapses and the composition of the neuropil, it was important to compare transplant with spinal cord. If regenerated DRG axons establish synapses within transplants, then comparing the types of synapses formed in transplants with those formed in normal spinal cord will provide a means for determining whether adult axons that regenerate into fetal spinal cord establish synaptic contacts according to rules similar to those that govern developing axons.

To compare the composition and synaptic organization of the transplant and spinal cord, we selected lamina I of the spinal cord for stereological and morphometric analyses. Although no area of spinal cord can be considered equivalent to the area of the transplant into which dorsal root axons regenerated, lamina I was chosen for the following reasons:

i) it is the lamina closest to the dorsal root entry zone and the majority of dorsal root axons regenerated into transplants terminate within 2 mm of the dorsal root-transplant interface;

ii) unlike other regions of normal spinal cord, both myelinated and unmyelinated dorsal root axons terminate within lamina I;

iii) lamina I is a region rich in calcitonin gene-related peptide (CGRP) (Rosenfeld et al., '83; Gibson et al., '84; Carlton et al., '87, '88; McNeill et al., '88; Chung et al., '88) and in which the CGRP is derived only from the large number of A delta and the C fibers that terminate there (Willis and Coggeshall, '78; Light and Perl, '79; Cervero, '86; Sugiura et al., '86); the region of the transplant within 2 mm of the dorsal root-transplant interface is also richly innervated with DRG axons immunoreactive for CGRP (Tessler et al., '88).

MATERIALS AND METHODS

Surgical procedures

Ten male and female adult (200–300 g) Sprague-Dawley rats (Zivic Miller, Allison Park, PA) were used as graft recipients. The rats were anesthetized with ketamine hydrochloride (95 mg/kg), xylazine (10 mg/kg), and acepromazine maleate (0.7 mg/kg), and laminectomies of the T13 or L1 vertebrae were performed with a speed drill (Dremel, Racine, WI) to expose the lumbar enlargement. After dorsal roots were sharply transected close to the dorsal root entry zone and reflected caudally, a hemisection cavity 3–4 mm long was created in the lumbar enlargement by gentle aspiration. Segments of spinal cord 5 mm long were dissected from embryonic day 14 (E14) or E15 Sprague-Dawley rat pups and introduced into the cavity according to procedures previously described (Reier et al., '86). The cut dorsal root stumps were juxtaposed to the transplants, the resected vertebral arch was replaced, and the wound was closed in layers. This procedure is similar to that described by Reier et al., ('86) and Tessler et al. ('88).

Anterograde WGA-HRP labeling procedure

One to 3 months after transplantation, 3 recipients were anesthetized as described above, the original wound was reopened, and the dorsal roots juxtaposed to the graft were identified. Following a procedure previously described (Beattie et al., '78; Tessler et al., '88), these roots were cut 5 mm distal to the entry site and the central end of the cut root was inserted into a tip of a micropipet filled with 2% WGA-HRP, where it remained for at least 1 hour. Forty-eight hours later the animals were deeply anesthetized with sodium pentobarbital (Nembutal, 40 mg/kg, i.p.) and per-

TABLE 1. Comparison of Composition of Neuropil (% of Area)

	Animal	Myelinated Axons	Unmyelinated Axons	Terminals	Perikarya & Dendrites	Non-neuronal Structures (% of glia)
Transplant	1	7.87	8.03	31.39	29.29	23.41 (97.18)
	2	16.53	10.18	22.77	25.26	25.26 (95.84)
	3	1.84	12.63	32.01	45.96	7.57 (92.21)
	4	9.85	18.40	30.86	35.69	5.20 (96.35)
Mean ± S.E.M.		9.02 ± 1.98	12.31 ± 1.46*	29.26 ± 1.42*	34.05 ± 2.86	15.36 ± 3.42 (96.40 ± 0.72)
Lamina I	1	10.44	28.05	18.80	37.56	5.15 (93.81)
	2	11.50	32.08	15.54	25.65	5.07 (95.46)
	3	10.31	26.02	13.18	42.45	8.05 (73.45)
	4	8.75	25.01	21.27	37.77	7.19 (84.56)
Mean ± S.E.M.		10.25 ± 0.57	27.79 ± 1.56	17.20 ± 1.78	35.86 ± 3.56	6.37 ± 0.75 (86.2 ± 5.05)

*Transplant differs significantly from lamina I at the $P < 0.05$ level using the Mann-Whitney two-sample test. The survival periods of each transplanted animal are as follows: animal 1, 1 month; animal 2, 2 months; animals 3 and 4, 3 months.

fused through the ascending aorta with 75–100 ml of physiological saline followed by 500 ml of a solution containing 1% paraformaldehyde, 2.5% glutaraldehyde, and 0.02 mM $CaCl_2$ in 0.1 M phosphate buffer (pH 7.4). The spinal segments that included the transplants were dissected out. Sections 50 μm thick were cut in the sagittal plane on a Vibratome and processed for HRP visualization using a solution of 0.05% 3,3'-diaminobenzidine (DAB), 0.01% hydrogen peroxide, and 0.01 M imidazole in 0.05 M Tris-HCl buffer, pH 7.4 according to the protocol of Straus ('82). Alternate sections from each animal were prepared for light and electron microscopic analyses. Tissues for light microscopic study were mounted on subbed slides and coverslipped, while specimens for electron microscopic evaluation were postfixed in a solution of 1% osmium tetroxide in 0.1 M Sym-collidine buffer for 20 min at 45°C (Ruda et al., '86), dehydrated, and flat-embedded in Epon-Araldite mixture.

CGRP immunocytochemistry

For light microscopic analysis, three host rats (1–3 months after transplantation) and three normal rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Spinal segments that contained transplants or normal L4 or L5 spinal cord were removed, sectioned on a cryostat (14 μm), and mounted on subbed slides. The sections were then processed for the peroxidase-antiperoxidase (PAP) technique according to methods that have been described previously (Sternberger, '86; Tessler et al., '80, '88).

For electron microscopic analysis, four recipients (1 to 3 months after transplantation) and four control animals were deeply anesthetized with sodium pentobarbital and perfused through the ascending aorta with 50 ml of physiological saline followed by 500 ml per animal of 0.1 M cacodylate buffer pH 7.4 (4°C) containing 3% paraformaldehyde, 3% glutaraldehyde, 0.1% picric acid, and 0.02 mM $CaCl_2$. Segments 5–6 mm long that contained transplants or normal spinal cord were immediately removed. Transplants were cut with a Vibratome (40 μm) in the sagittal plane because CGRP-immunoreactive fibers were more easily detected there than in the coronal plane. To study CGRP-immunoreactive fibers in lamina I, specimens of spinal cord dissected from control animals were sectioned in the coronal plane (40 μm). All sections were incubated in 1% sodium borohydride in phosphate buffer saline (PBS), pH 7.4, for 30 min and rinsed several times in PBS (Kosaka et al., '86; Carlton et al., '87; Carlton et al., '88; McNeill et al., '88). The specimens were then treated with a series of ascending and descending concentrations of ethyl alcohol in PBS as previously described (Light et al., '83; Carlton et al., '87) and processed for the PAP method of Sternberger ('86). After rinsing with normal goat serum (NGS) diluted 1:30, sections were incubated overnight at room temperature in primary antiserum (Peninsula Laboratories, Belmont, CA) diluted 1:16,000 with a solution of PBS containing 1% NGS. After washing twice in 1% NGS, the sections were incubated in goat anti-rabbit IgG diluted 1:50 for 90 minutes at room temperature, and then rinsed in 1% NGS. The specimens were incubated in PAP (1:100) for 90 minutes at room temperature, then washed in PBS followed by 0.05 M Tris-HCl buffer (pH 7.4) and incubated in 0.05% DAB containing 0.01% hydrogen peroxide diluted in 0.05 M Tris-HCl buffer for 6–8 minutes at room temperature. After fixation in 3% glutaraldehyde solution for 2 hours, the sections were osmicated (Ruda et al., '86), dehydrated and flat-embedded.

The characteristics of the primary antisera against CGRP and controls for the specificity of staining have been described in detail previously (Tessler et al., '88).

Stereological analysis

Because CGRP-immunoreactive fibers were too extensively distributed within the transplants to study every fiber, the following sampling procedure was used. From each recipient two or three flat-embedded Epon sections were selected that contained CGRP-labeled fibers arborizing either within 1 mm of the dorsal root-transplant interface (animals 1 and 2 in Table 1) or within 1–2 mm of the interface (animals 3 and 4 in Table 1). These areas were considered to be analogous to lamina I. Ultrathin sections were cut, placed on 400 mesh grids, and examined in a JEOL 100S electron microscope. Neither uranyl acetate nor lead citrate staining was used. Sections were outlined on graph paper at

Fig. 1. Sagittal sections of embryonic spinal cord grafts 1 month after transplantation. Bar = 100 μm. a: Host dorsal root (DR) axons labeled with 2% WGA-HRP regenerate into the transplant (TP) and arborize within the graft. Axons show numerous varicosities and some end in globular dilatations (arrows). b: CGRP-immunoreactive axons are shown in host dorsal root (DR) and transplant (TP). Regenerated axons contain varicosities along their paths and are tangled together to form dense plexuses (*). Inset shows relationship of transplant (TP) and host spinal cord (H) and the interface (arrowheads) between dorsal root (DR) and transplant. Stained with chromoxane cyanine R and cresyl violet (Clark, '81). Bar = 500 μm.

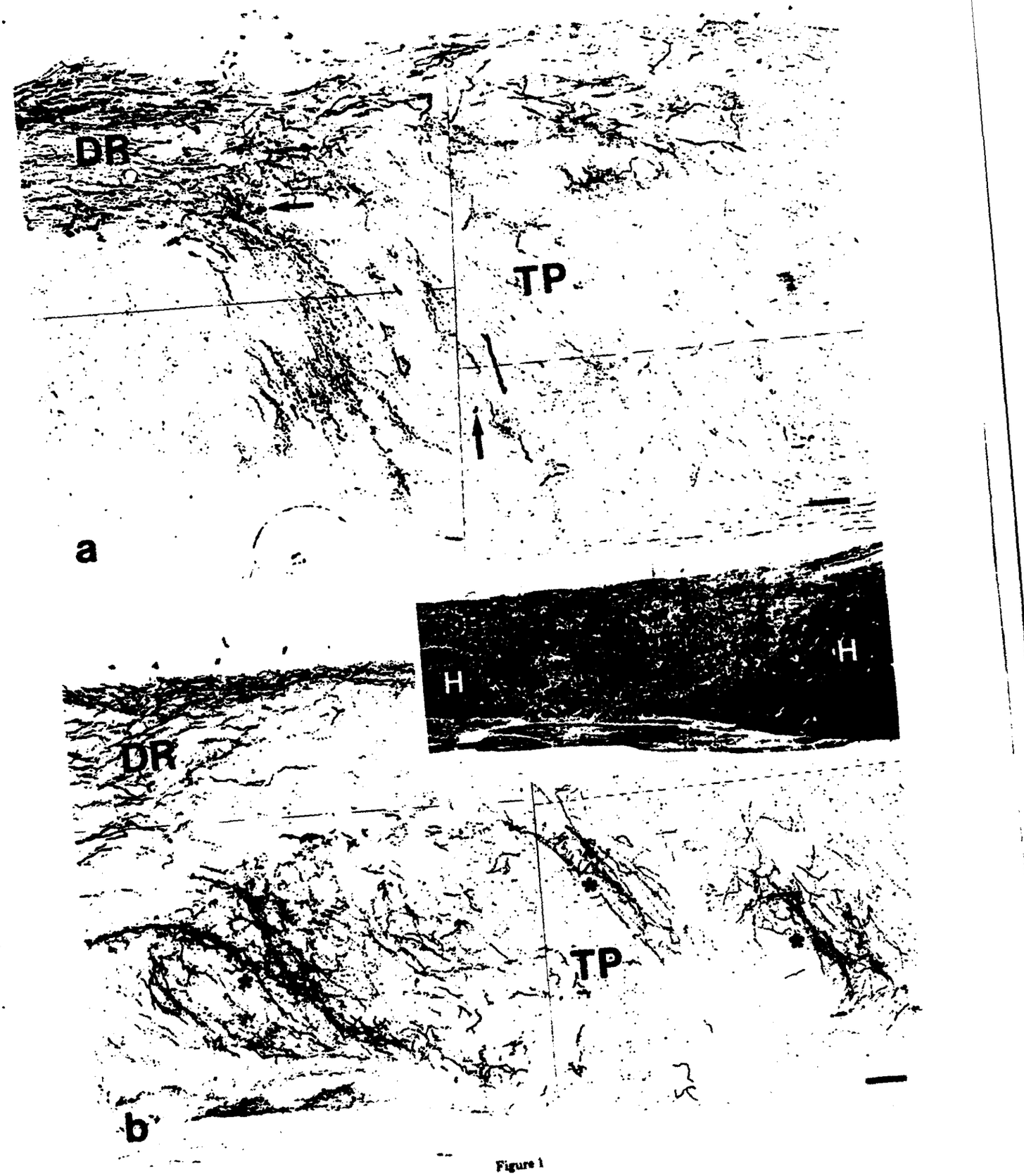

Figure 1

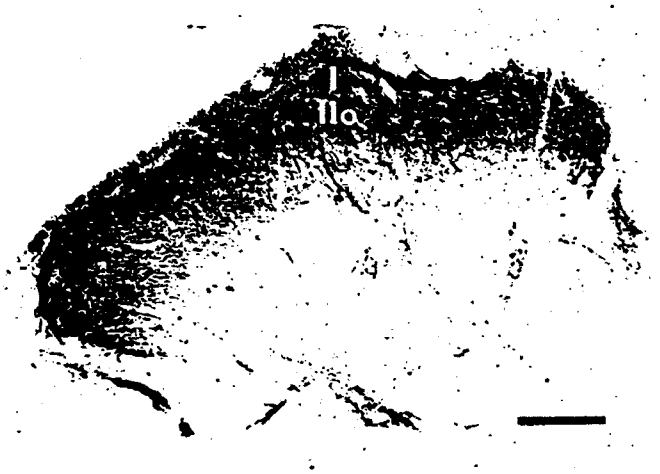

Fig. 2. CGRP-immunoreaction product in transverse section of normal rat lumbar spinal cord (L4 segment). Labeled axons and terminals are evenly distributed in laminae I and IIo. Bar = 100 μm.

low magnification, and regions were identified in which at least 10 adjacent grid squares contained profiles immunoreactive for CGRP. Two photographs were randomly taken at a ×8,000 magnification from alternate grid squares for a total of 40–50 photographs for each recipient. Grid squares in which more than one-half of the field was occupied by blood vessels or by bundles of myelinated axons were excluded from the survey (Murray and Goldberger, '86). Axons (myelinated and unmyelinated), terminals, cell bodies, dendrites, and nonneuronal structures (glia, blood vessels, and unidentified profiles) were identified using criteria described previously (Lemkey-Johnston and Larramendi, '72; Murray and Goldberger, '86), and the area fraction occupied by each structure was measured on electron micrographs at a final magnification of ×20,000. In brief, these criteria were as follows:

i) nerve terminals: bulbous structures containing a cluster of 5 or more synaptic vesicles;

ii) unmyelinated axons: membrane-bounded structures with pale cytoplasm that included many profiles of microtubules and/or mitochondria;

iii) dendrites: irregularly shaped structures with a pale cytoplasmic matrix;

iv) glial structures: irregularly shaped processes that contained bundles of glial filaments (astroglia) or dark cytoplasmic matrix (oligodendroglia).

The area fraction occupied by axons and terminals was further classified into CGRP-labeled and CGRP-unlabeled elements as determined by the presence or absence of electron-dense immunoreaction product.

The stereological analysis of four normal control animals was performed according to the protocol of Murray and Goldberger ('86). Thin cross-sections containing an entire lamina I were cut and placed on 400 mesh grids. At low magnification, the sections were outlined on graph paper, and landmarks were identified using camera lucida drawings of Epon-embedded 1-μm thick cross-sections stained with 0.7% toluidine blue. The lamina I-II boundary was determined according to criteria previously described (Ralston, '68; McClung and Castro, '76; Ralston and Ralston, '79; Snyder, '82; Molander et al., '84; Murray and Goldberger, '86). In areas in which the profiles of lamina I covered 10 to 15 grid squares, two electron micrographs were taken at a magnification of ×8,000 from each grid square so as to include an entire marginal zone. Structures were then classified and the area fractions calculated using the same procedures as described above for the transplants.

To determine the synaptic density within transplants and lamina I, the number of synapses was counted according to the protocol of Sørensen and Zimmer ('88). The area examined on electron micrographs corresponded to 9.0 μm × 7.2 μm and the synaptic density was expressed as the number of synapses per 100 μm^2. Synapses were also classified as CGRP-labeled or CGRP-unlabeled.

Morphometric analysis

Synaptic terminals in electron micrographs used for the stereological analysis were subsequently analyzed morphometrically. A profile was considered to be a synaptic terminal if it contained a cluster of five or more synaptic vesicles, formed unequivocal synaptic complexes with neuronal profiles, and had vesicles closely associated with the synaptic junctions. The area, perimeter, longest dimension, length of synaptic contacts, and glial profile length per perimeter of 60–120 synaptic terminals from each transplanted and control animal were measured using the Bioquant System IV (R&M Biometrics, Inc., Nashville, TN). Because lamina I of normal animals was sectioned transversely whereas transplants were sectioned longitudinally, we determined whether the shape of the synaptic terminals was affected by the plane of section by calculating the shape factor (Mize, '85) according to the formula 4π area/perimeter.^{2} The mean shape factor of CGRP-immunoreactive synaptic terminals in transplants was 0.46 ± 0.04 (S.E.M.) and in lamina I, 0.52 ± 0.02. Since there was no statistically significant difference between the two ($P > 0.05$, the Mann-Whitney two-sample test), these data indicated that the shape of the profiles of at least the CGRP-immunoreactive synaptic terminals was unaffected by the different section planes used in transplanted and control animals. Each synapse from normal lamina I and transplants was further studied and classified for the following characteristics:

i) CGRP immunoreactivity;

ii) synaptic vesicle type: synapses were classified as containing spherical, spherical and dense cored, or pleomorphic vesicles;

iii) number of synaptic contacts: a terminal was classified as simple if it made only one synaptic contact, or complex, if it made two or more synaptic contacts per profile. A multisynaptic index (MSI) was calculated after determining the number of contacts with separate postsynaptic structures per terminal profile.

iv) type of postsynaptic structures: a synapse was classified as axodendritic, axosomatic, or axoaxonic.

Since the distinction between axon terminals and dendritic terminals containing vesicles is difficult (Peters et al., '76), the following criteria were used to identify axoaxonic synapses: 1) presynaptic element is recognized as an axon; 2) synaptic vesicles contained in presynaptic elements are associated closely with the junction; 3) postsynaptic elements

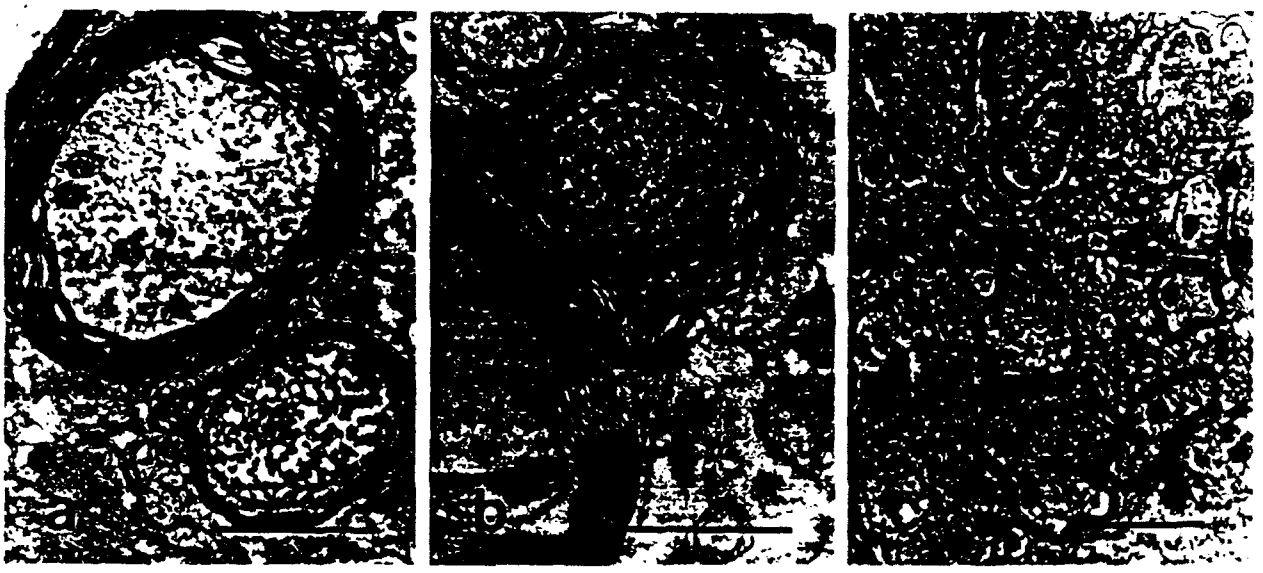

Fig. 3. Electron micrographs of labeled axons in transplants (a and b) and lamina I (c). Bar = 0.5 μm. a: Myelinated axon labeled by WGA-HRP has regenerated within transplant. b: An example of a CGRP-immunoreactive myelinated axon found within transplants. c: One myelinated (*) and two unmyelinated axons (arrows) contain CGRP-immunoreactivity associated with microtubules and axolemma.

contain synaptic vesicles and/or microtubules labeled for CGRP; and 4) junctions have the characteristics of normal synapses.

The stereological data were analyzed statistically by the Mann-Whitney two-sample test. The significance of the morphometric analysis was evaluated by one-way ANOVA ($P < 0.05$). If there were significant differences between transplant and lamina I, each result was corrected for multiple comparisons using the Duncan test. All statistical analyses were performed using the Number Cruncher Statistical System (Dr. Jerry L. Hintze, Kaysville, UT).

RESULTS

Light microscopy

Transplant. Anterograde WGA-HRP labeling of the severed host dorsal root reveals that regenerated fibers have grown into each of the three transplants studied. Labeled axons course longitudinally within the dorsal roots, appear to turn at the dorsal root-transplant interface, and grow across the entire dorsal-ventral width of the transplants. The regenerating dorsal root axons arborize extensively and radially within the grafts and contain varicosities along their length. Most of the endings of labeled fibers show globular dilatations that vary in size (Fig. 1a).

Regenerating axons immunoreactive for CGRP are more numerous within the transplants and arborize more widely than those seen following labeling with WGA-HRP. In some sections, CGRP-immunoreactive axons appear to be tangled together to form dense plexuses. CGRP-immunoreactive fibers show varicosities along their paths, but dilatations of axon endings are less prominent than after HRP filling (Fig. 1b). These observations are very similar to those that we have previously reported (Tessler et al. '88).

Superficial dorsal horn. When studied in cryostat sections or in semithin (1 μm) epon sections, CGRP-immunoreactive fibers bearing numerous varicosities are found in Lissauer's tract and laminae I and II outer (IIo) (Fig. 2). Immunoreactive fibers are evenly distributed in lamina I, traverse a layer of large flattened marginal neurons (Waldeyer cells), and are present throughout lamina IIo.

Electron microscopy

Transplant. In graft recipients whose dorsal roots were labeled with WGA-HRP, regenerating axons and terminals are readily identified by their dense content of HRP reaction product. Labeled myelinated axons (average diameter 1.67 μm) (Fig. 3a) are observed only close to the dorsal root-transplant junction, where some of their terminals form synaptic contacts upon dendritic profiles. Most of these synaptic complexes are asymmetric (type 1, Gray '59) with parallel pre- and postsynaptic membranes, presynaptic accumulation of spherical vesicles (mean diameter 45 nm), and postsynaptic thickenings (Fig. 4a). All of the synaptic terminals that we observed to be labeled by WGA-HRP transport contain spherical vesicles.

CGRP-immunoreactive axons and terminals are also recognized within the grafts. In axons, CGRP-labeling is mainly associated with microtubules (Fig. 3b); in axon terminals, with dense-cored vesicles (Fig. 4b,c). Many CGRP-labeled terminals make one or two synaptic contacts with postsynaptic profiles, but some endings form three or more synapses. Although these synapses are similar morphologically to those labeled by WGA-HRP, the majority of CGRP-immunoreactive presynaptic endings contain both spherical and dense cored vesicles (Fig. 4b,c). Some labeled synaptic terminals are entirely surrounded by glial processes (Fig. 5), however, most are only partially enveloped.

In general the morphology of regenerated axons and terminals identified by the two labeling methods closely resembles that of the corresponding structures found in normal lamina I. Two types of unusual profiles, however, occur

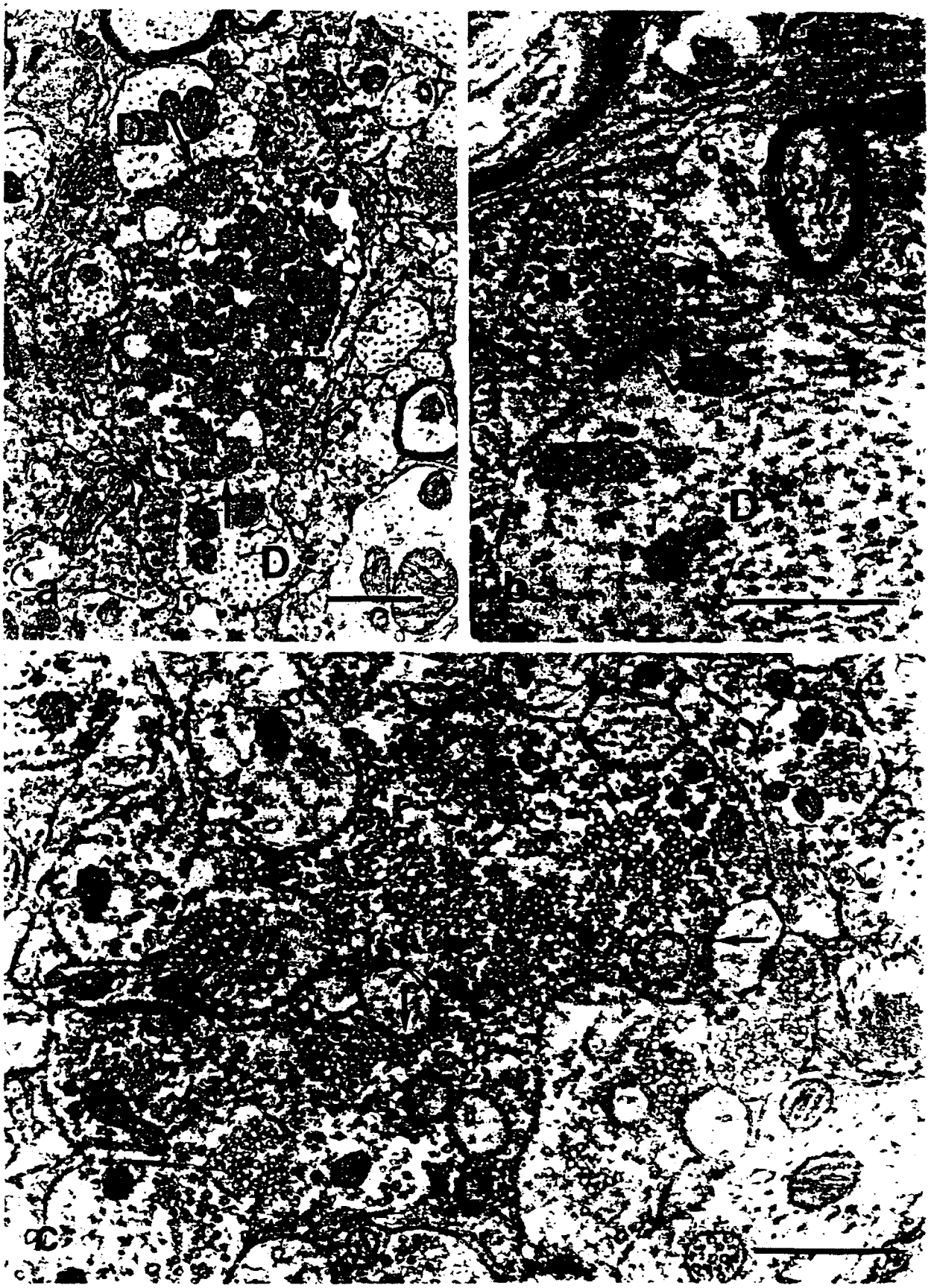

Figure 4

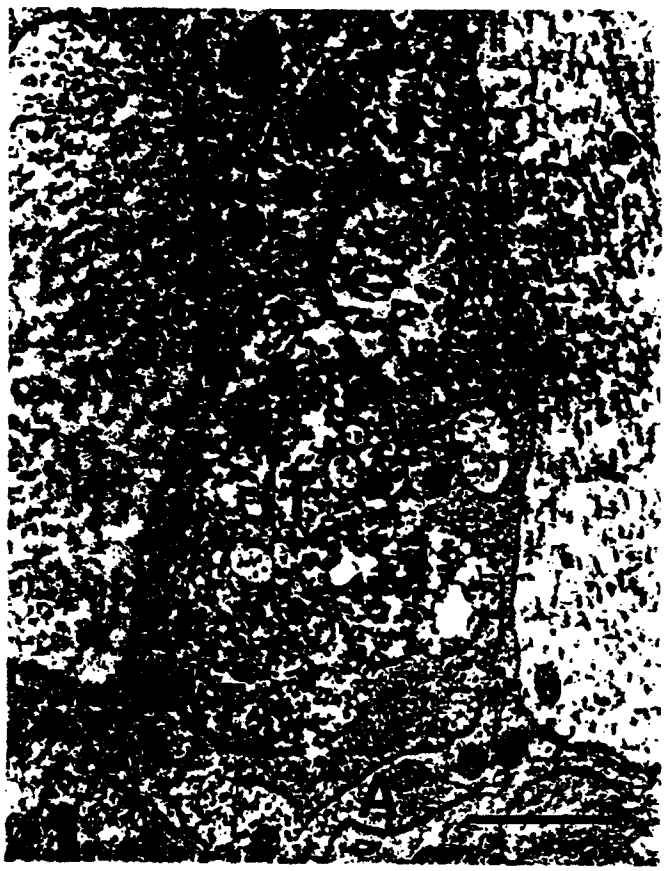

Fig. 5. In the region close to the dorsal root-transplant interface, several closely approximated CGRP-labeled axon terminals (T) are densely surrounded by astrocytic processes (A). Bar = 1 μm.

within the grafts. One type consists of varicosities filled with CGRP-immunoreactive dense-cored vesicles that are composed of several overlapping and apparently interconnecting axonal endings (Fig. 6a). These varicosities occur close to the dorsal root-transplant interface and are densely covered with glial profiles. The second type consists of large terminals that are apposed to each other by atypical junctions. These are labeled by WGA-HRP but only infrequently by CGRP. The longest dimension of these large terminals is approximately 4 μm, and they are filled with numerous spherical clear vesicles, mitochondria, and microtubules (Fig. 6b). Similar dilated terminals have been reported during the early stages of DRG axon regeneration into adult spinal cord (Stensaas et al., '79; Reier et al., '83a; Liuzzi and Lasek, '87), and in this respect the structures resemble growth cones. However, the enlarged terminals that we observed are apposed to each other with unusual junctions consisting of parallel thickened membranes separated by a variable (25–50 nm) interval, and the vesicles are only in some instances closely associated with the membranes. When studied by interrupted serial sections, the large terminals appear to make no definite synaptic contacts with other profiles in the neuropil. These observations therefore suggest that the structures more closely resemble regenerating axons of adult DRG neurons whose growth has been arrested (Stensaas et al., '79; Reier et al., '83a; Liuzzi and Lasek, '87) than those that are forming growth cones (Pomerat et al., '67; Bunge et al., '83).

Lamina I. CGRP-immunoreactive axons and synaptic terminals are distributed throughout lamina I, but fewer labeled axons, especially myelinated axons, are found in the marginal zone than in Lissauer's tract. In both myelinated (average diameter 1.50 μm) and unmyelinated axons, reaction product is most consistently associated with microtubules, but in heavily labeled profiles it is also associated with the axolemma and mitochondria (Fig. 3c). Most CGRP-immunoreactive terminals contain two types of synaptic vesicles: numerous small, clear, spherical vesicles (mean 50 nm in diameter) and less numerous large, spherical dense-cored vesicles (mean 95 nm in diameter). Reaction product in terminals is found in the cores of dense-cored vesicles. The majority of labeled synaptic terminals make one or two synaptic contacts with dendritic profiles that lack vesicles (Fig. 7a,b), but some terminals form three or more synapses with postsynaptic profiles. These observations are similar to the findings of McNeill et al. ('88).

Stereological analysis

Composition of neuropil (Table 1, Fig. 8). Perikarya and dendrites occupy 34% and axon terminals occupy 29% of the area fraction in the transplant regions examined, while myelinated axons occupy 9% and unmyelinated axons occupy 12%. The area fraction occupied by most of the structures that we analyzed varies little among the transplants, but the amount occupied by astrocytic profiles is variable and depends on the portion of the transplant that is sampled. Within 1 mm of the dorsal root-transplant interface (animals 1 and 2 in Table 1), astrocytes occupy approximately 24%, whereas in the region 1–2 mm from the interface (animals 3 and 4 in Table 1) their percentage of the area fraction is approximately 6%. In lamina I, perikarya and dendrites occupy 36% and unmyelinated axons 28% of the area fraction, whereas axon terminals constitute 17%, myelinated axons 10%, and glia 6%. The area fractions occupied in transplants by perikarya and dendrites and by myelinated axons are therefore very similar to those observed in lamina I. The area occupied by glia within the transplants is the same as that occupied in lamina I. At the dorsal root-transplant interface, glia occupy a percentage of the area 4 times larger than in lamina I.

CGRP-labeling of axons and terminals (Table 2). The stereological analysis of axons and terminals labeled for CGRP shows the percentage of the neuropil that is occupied by structures derived only from dorsal roots. In transplants, axon terminals represent 29% of the area fraction, unmyelinated axons 13%, and myelinated axons 1%. In lamina I, axon terminals occupy 17% of the area fraction, unmyelinated axons 13%, and myelinated axons 5%. The area fraction occupied by CGRP-unmyelinated axons is therefore not different in transplants and lamina I, whereas

Fig. 4. Electron micrographs from transplants. Bar = 1 μm. a: A WGA-HRP-labeled complex presynaptic terminal containing spherical vesicles makes asymmetric synaptic contacts (arrows) upon different dendritic profiles (D). b: A typical asymmetric axodendritic synapse (arrow) in which a CGRP-labeled presynaptic profile contacts a dendrite (D). c: An example of a CGRP-immunoreactive complex terminal containing spherical and dense-cored vesicles that makes asymmetric synaptic contacts (arrows) upon different dendritic profiles. This terminal is surrounded by numerous vesicle-containing profiles.

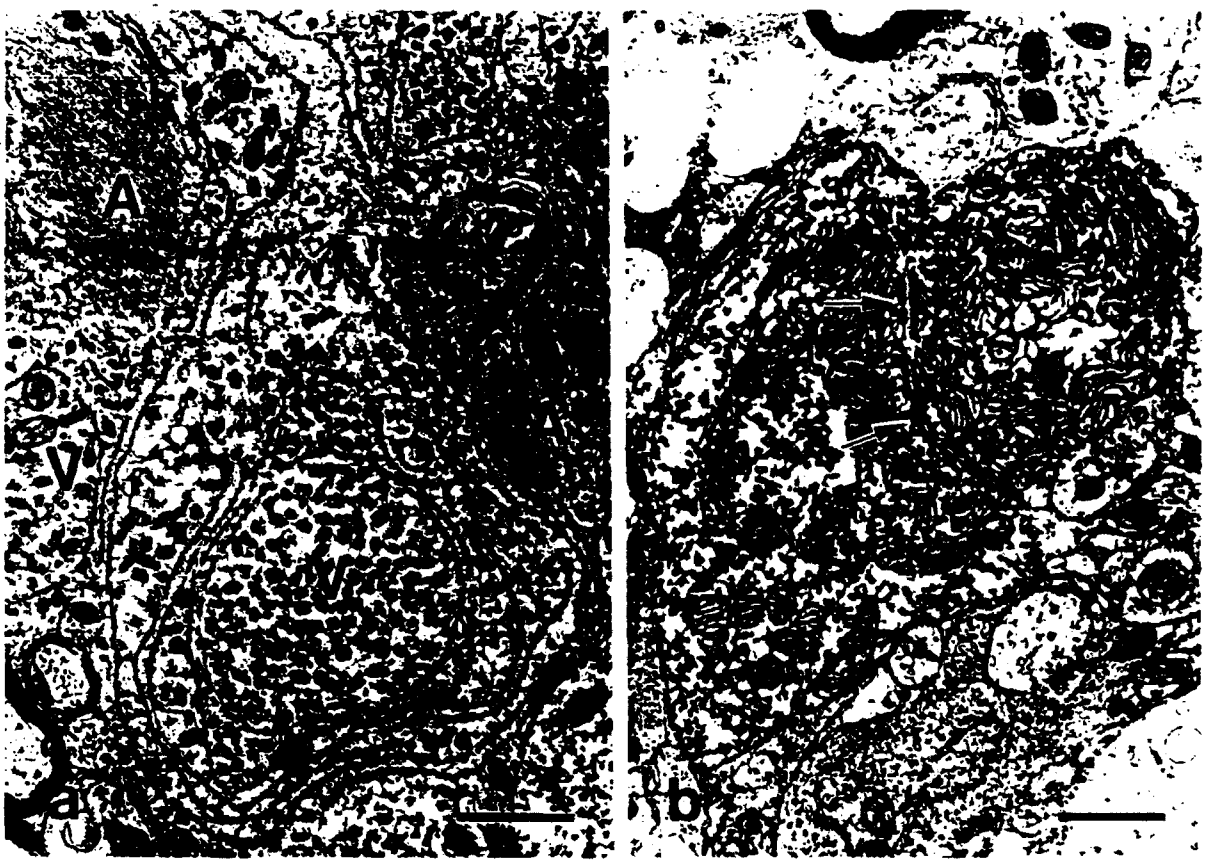

Fig. 6. Electron micrographs of unusual structures within transplants. Bar = 1 μm. a: An example of unusual dilated varicosities (V) consisting of several closely apposed structures that are filled with CGRP-labeled and, -unlabeled dense-cored vesicles. Astrocytic processes (A) surround these structures. b: Large terminals labeled by WGA-HRP that contain numerous spherical vesicles, mitochondria, and microtubules. These endings are characterized by unusually long cell junctions separated by 20–50 nm (arrows).

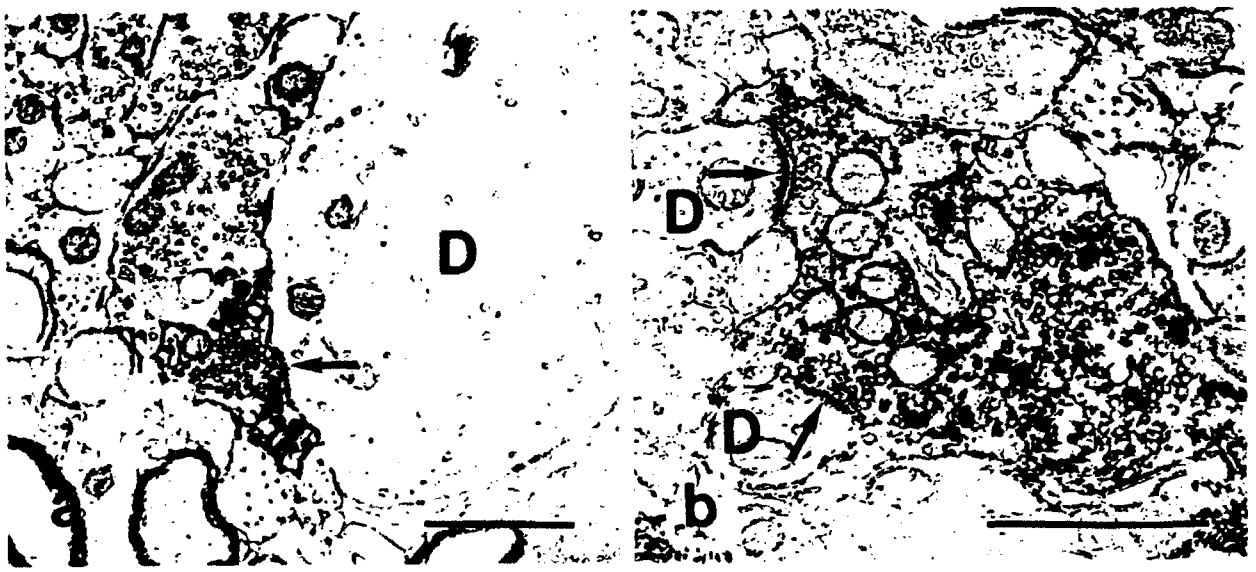

Fig. 7. Electron micrographs illustrating CGRP-immunoreactive terminals in normal lamina I. Bar = 1 μm. a: An example of a simple terminal that contains labeled spherical and dense-cored vesicles, and makes an asymmetric contact (arrow) upon a dendritic profile (D). b: An example of a complex terminal that contains immunoreactive spherical and dense-cored vesicles, and makes two asymmetric contacts (arrows) upon different dendritic profiles (D).

TABLE 2. Comparison of % CGRP-Labeling of Axons and Terminals[1]

	N	Myelinated Axons	Unmyelinated Axons	Terminals	SV[2] (% area)	SV[2] & DCV[3] (% area)	PV[4] (% area)
Transplant	4	1.04 ± 0.60*	13.06 ± 5.31	29.36 ± 2.56*	20.79 ± 2.24 (84.68 ± 2.21)	78.51 ± 5.24 (14.50 ± 2.18)	16.67 ± 10.91 (0.81 ± 0.24)
Lamina I	4	5.36 ± 1.32	12.76 ± 1.38	21.14 ± 1.67	16.46 ± 1.26 (89.84 ± 2.07)	72.93 ± 3.05 (7.36 ± 2.54)	7.82 ± 5.16 (1.73 ± 0.34)

[1]Values are mean ± S.E.M.
[2]Spherical vesicles
[3]Dense-cored vesicles
[4]Pleomorphic vesicles
*Transplant differs significantly from lamina I at the $P < 0.05$ level using the Mann-Whitney two-sample test.

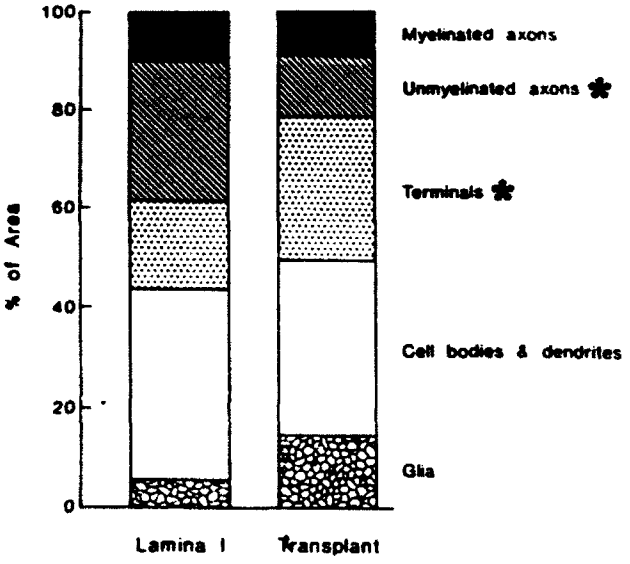

Fig. 8. Composition of neuropil. Histograms summarizing stereological data from lamina I (N = 4) and transplants (N = 4). Bars are divided according to the area fraction occupied by myelinated and unmyelinated axons, terminals, perikarya and dendrites, and glia. Areas occupied by unmyelinated axons and terminals in transplants are significantly different from lamina I (*).

the percentage of area occupied by CGRP-labeled myelinated axons in transplants is significantly decreased compared with lamina I and the area fraction occupied by CGRP-labeled dorsal root axon terminals is increased.

Synaptic density (Table 3). The total number of synapses per 100 μm^2 in transplants is 5.78. This total includes 2.22 that are immunoreactive for CGRP and 3.57 that are unlabeled. In lamina I the total synaptic density is 7.72 per 100 μm^2, of which 2.43 are labeled for CGRP and 5.29 are unlabeled. The density of synapses in which the presynaptic endings are labeled for CGRP is therefore the same in transplants as in lamina I, whereas both the total synaptic density in transplants and the density of synapses in which the presynaptic terminals are unlabeled for CGRP are significantly reduced.

Synaptic terminals

Synaptic vesicles (Table 2). Nearly 85% of axon terminals in transplants contain only spherical vesicles. Much smaller percentages of the remaining terminals contain both

TABLE 3. Comparison of Synaptic Density[1]

	N	CGRP-L[2]	CGRP-UL[3]	Total
Transplant	4	2.22 ± 0.45	3.57 ± 0.36*	5.78 ± 0.77*
Lamina I	4	2.43 ± 0.12	5.29 ± 0.42	7.72 ± 0.36

[1]No. of synapses per 100 μm^2 (mean ± S.E.M.).
[2]No. of synapses in which presynaptic terminals are labeled for CGRP
[3]No. of synapses in which presynaptic terminals are not labeled for CGRP
*Transplant differs significantly from lamina I at the $P < 0.05$ level using the Mann-Whitney two sample test.

spherical and dense cored vesicles or pleomorphic vesicles. Although constituting only 7% of the total area occupied by axon terminals, those that contain both spherical and dense cored vesicles represent nearly 75% of the terminals that contain CGRP. Similar results are found in lamina I, where 90% of axon terminals contain only spherical vesicles and the area fraction that each synaptic type occupies is not significantly different from the area fraction that it occupies in transplants. In lamina I, as in transplants, terminals that contain both spherical and dense cored vesicles represent a small percentage of the total area occupied by terminals, but include nearly 75% of the terminals immunoreactive for CGRP.

Morphological features (Tables 4, 5). CGRP-containing terminals are significantly larger than unlabeled terminals in transplants when area, perimeter, and longest dimension are measured (Table 4). The average length of the synaptic contacts formed by CGRP-immunoreactive terminals in transplants is also significantly longer than that of unlabeled terminals (Table 5). Glia cover a similar percentage of the perimeter of both labeled and unlabeled terminals (Table 4). In lamina I, CGRP-containing synaptic terminals are also significantly larger than synaptic terminals unlabeled for CGRP, but the length of synaptic contact formed by labeled and unlabeled terminals is the same and a similar percentage of the perimeter of both types of terminals is covered by glia. Both labeled and unlabeled terminals in transplants are significantly larger than their counterparts in lamina I, and the length of synaptic contact established by CGRP-immunoreactive terminals in transplants exceeds that found in lamina I. The length of synaptic contacts formed by terminals that lack CGRP is not significantly different in transplants and lamina I, and the percentage of the perimeter of synaptic terminals that is covered with glia shows no significant changes between lamina I and transplants (Table 4). Therefore, although the results of the stereological analysis show that the amount of glia varies among transplants (Table 1), this variability does not occur in association with the synaptic terminals.

TABLE 4. Comparison of Synaptic Terminals[1]

	N	Group	Area (μm^2)	Perimeter (μm)	Longest axis (μm)	Glia per perimeter (%)
Transplant	4	CGRP-L[2] (A)	2.05 ± 0.18	7.61 ± 0.61	2.46 ± 0.17	38.80 ± 3.40
		CGRP-UL[3] (B)	1.00 ± 0.09	4.44 ± 0.19	1.50 ± 0.08	43.13 ± 5.99
Lamina I	4	CGRP-L[2] (C)	1.16 ± 0.09	5.26 ± 0.28	1.67 ± 0.08	32.13 ± 7.22
		CGRP-UL[3] (D)	0.75 ± 0.03	3.99 ± 0.23	1.29 ± 0.07	36.68 ± 11.00
Significant differences among groups*:			Area, perimeter, and long axis: Glia per perimeter:			A > C > B > D None

[1]Values are mean ± S.E.M.
[2]CGRP-labeled synaptic terminals
[3]CGRP-unlabeled synaptic terminals
*Overall significance determined by one way ANOVA ($P < 0.05$) and individual post hoc comparisons are with the Duncan test corrected for multiple comparison ($P < 0.0001$).

TABLE 5. Comparison of Synaptic Contacts[1]

	N	Group	Length of SC[2] (μm)	Type (%)		MSI[3]	Postsynaptic structure (%)		
				Simple	Complex		Axodendritic	Axosomatic	Axoaxonic
Transplant	4	CGRP-L[4] (A)	0.73 ± 0.06	76.36 ± 7.01	23.65 ± 7.01	1.34 ± 0.11	70.97 ± 4.92	10.36 ± 2.06	18.66 ± 2.91
		CGRP-UL[5] (B)	0.53 ± 0.01	90.44 ± 2.77	9.56 ± 2.77	1.11 ± 0.04	88.39 ± 1.08	5.54 ± 1.17	6.08 ± 0.92
Lamina I	4	CGRP-L[4] (C)	0.56 ± 0.02	74.71 ± 5.52	25.30 ± 5.52	1.36 ± 0.06	96.02 ± 0.56	4.45 ± 0.26	0.53 ± 0.63
		CGRP-UL[5] (D)	0.47 ± 0.01	90.73 ± 2.96	9.27 ± 2.96	1.10 ± 0.03	94.30 ± 1.37	5.57 ± 1.35	0.23 ± 0.23
Significant differences among groups[6]:			Length of SC[1]: Simple synaptic terminals: Complex synaptic terminals: MSI[1]:		A > C, B, D** None None C, A > B, D*		Axodendritic: Axosomatic: Axoaxonic:	C, D, B > A*** A > B = D, C* A > B > C, D***	

[1]Values are mean ± S.E.M.
[2]Synaptic contacts.
[3]Multisynaptic index.
[4]CGRP-labeled synaptic terminals.
[5]CGRP-unlabeled synaptic terminals.
[6]Overall significance determined by one way ANOVA ($P < 0.05$) and individual posthoc comparisons are with the Duncan test corrected for multiple comparison at the $P < 0.05$(*), $P < 0.001$(**), and $P < 0.0001$(***) levels.

Synaptic contacts (Table 5). In transplants, the majority of both CGRP-labeled terminals and terminals that lack CGRP form synaptic contacts with one profile (simple). However, nearly 25% of CGRP-labeled terminals contact more than one profile (complex) and this percentage is far greater than that of the complex contacts formed by unlabeled terminals. In lamina I, most labeled and unlabeled synapses are also simple, and a considerably larger percentage of labeled terminals than unlabeled are complex. The percentage of axon terminals that forms simple or complex synaptic contacts shows very little difference between transplants and lamina I, whether this is determined for CGRP-labeled terminals or for terminals that lack CGRP. In both tissues approximately 25% of labeled terminals are complex. Because the ratio between simple and complex synaptic terminals is unchanged in transplants and lamina I, the MSI also shows no significant alterations.

Most CGRP-labeled and CGRP-unlabeled terminals in transplants make contacts with dendritic processes. Some of these dendrites contain spherical and dense-cored vesicles, or pleomorphic and dense-cored vesicles (Fig. 9a) and are therefore difficult to distinguish from axons. A similar finding has been reported previously (Vidal-Sanz et al., '87; Carter et al., '88; Bray and Aguayo, '89). Approximately 10% of CGRP-immunoreactive synaptic terminals contact perikaryal profiles (Fig. 9b) and 19% form asymmetric contacts with axonal profiles (Fig. 10a,b). In lamina I, nearly 95% of both labeled and unlabeled synaptic terminals contact dendrites, and virtually all of the remaining synapses are axosomatic. The percentage of CGRP-immunoreactive synapses formed with dendrites is therefore significantly reduced in transplants compared with lamina I, and a significantly greater percentage of labeled synapses in transplants are axosomatic or axoaxonic. The percentage of CGRP-unlabeled synaptic terminals with axoaxonic synapses (Fig. 10c) is also increased in transplants but to a smaller extent.

DISCUSSION

Few injured primary afferent fibers of adult mammals penetrate the dorsal root entry zone and regenerate into spinal cord (Stensaas et al., '79; Reier et al., '83a; Bignami et al., '84; Reier et al., '89). Upon reaching the PNS-CNS interface, most either are reflected back toward the DRG or form enlarged blind endings on astrocytes and cease growth (Reier et al., '83a, '89; Liuzzi and Lasek, '87). Fetal spinal cord provides an environment that either supports or enhances the regeneration of mature DRG axons, since transected dorsal root axons of adult rats regrow for several millimeters into transplants (Tessler et al., '88). The major finding of the present study is that adult DRG axons not only regenerate into the transplants but also form synapses on neurons within the transplants.

Synapses in fetal spinal cord transplants have been reported previously (Reier et al., '83b, '85, '86; Inoue et al., '88; Jakeman et al., '89). Since the source of these synapses was not determined, it is not known if they resulted from regeneration of host axons or from neurons intrinsic to the transplant. In the present study we used two different labeling methods to identify the synapses established by DRG axons regenerated into transplants. Injury filling with WGA-HRP presumably labels some of each of the subpopulations of DRG axons that compose the DRG (Beattie et al., '78; Tessler et al., '88). CGRP-labeling shows a subset of

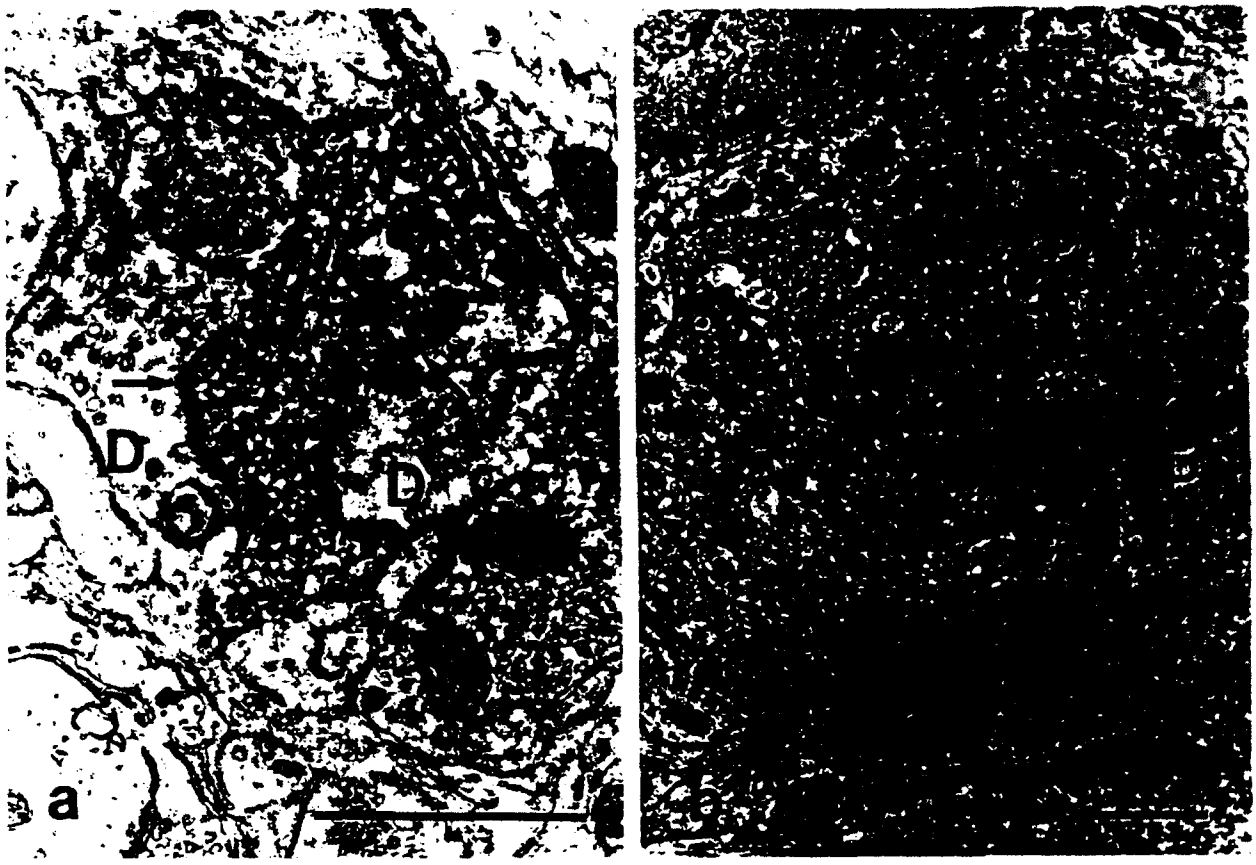

Fig. 9. CGRP-immunoreactive terminals forming axodendritic (a) and axosomatic (b) synapses within the transplants. Bar = 1 μm. a: Presynaptic terminal containing CGRP-labeled dense-cored vesicles makes asymmetric synaptic contacts (arrows) on dendrites containing vesicles (D). b: CGRP-immunoreactive presynaptic endings make contacts (arrows) upon perikaryon (P).

DRG axons. In normal dorsal horn CGRP originates exclusively from DRG neurons (Gibson et al., '84; Chung et al., '88), where it is principally associated with small neurons that give rise to A delta and C fibers (Cervero and Iggo, '80; Gibson et al., '84). As in normal spinal cord, CGRP in transplants is also derived from regenerated dorsal roots (Tessler et al., '88) and therefore from a subclass of unmyelinated (C) and finely myelinated (A delta) DRG axons.

Comparison of transplant neuropil with neuropil of lamina I

We selected lamina I of normal spinal cord for comparison with transplants because both i) receive input from dorsal roots; ii) contain finely myelinated and unmyelinated DRG axons; and iii) receive dense innervation by CGRP-immunoreactive dorsal roots. It must be recognized that, despite these similarities, the two regions cannot be considered entirely equivalent. Differences between transplants and lamina I may therefore reflect either unique features of the transplants or an organization characteristic of areas of spinal cord other than lamina I. Therefore, without a stereological analysis of all laminae of the spinal cord, which is beyond the scope of this study, differences between lamina I and transplants must be interpreted with caution. However, the major goal of our comparison of the two areas was to indicate the extent to which synapses and neuropil of the transplants can achieve an organization similar to that of a region of normal spinal cord in which DRG axons normally terminate. Thus, similarities between the transplant and lamina I are significant in their implications for the therapeutic promise of the transplant technique and are not subject to the difficulties inherent in interpreting differences between the two areas. In our discussion we therefore focus on the similarities between the transplants and lamina I.

Except for the presence of differentiated regions that resemble substantia gelatinosa (Reier et al., '83b, '85, '86; Jakeman et al., '89), the lamination patterns of spinal cord transplants are not apparent and their overall structure does not resemble that of normal spinal cord (Reier et al., '86). Similarities between the neuropil of transplants and normal spinal cord have been reported (Inoue et al., '88), however, and the present quantitative analysis extends these observations. The area fractions occupied by perikarya and dendrites and by myelinated axons are the same in transplants and lamina I. The area fractions occupied by astrocytes within transplants and lamina I also does not differ. Although the donor spinal cord is isolated from afferents at the time of grafting, the interior of the transplant is thus not gliotic. This lack of gliosis contrasts with the

appearance of the denervated neuropil of lamina II of the adult dorsal horn, which remains permanently gliotic after dorsal rhizotomy (Murray and Goldberger, '86).

A continuous layer of astroglial cytoplasm forms at the dorsal root-transplant interface, where the area fraction occupied by glia is increased by approximately 300% over that in normal spinal cord. Glial scars have been reported at the interface between transplant and host both in the spinal cord (Reier et al., '85, '86; Houlé and Reier, '88) and elsewhere in the CNS (Krüger et al., '86). If reactive astrocytes can inhibit the regeneration of DRG axons at the PNS-CNS transition zone (Reier et al., '83a, '89; Liuzzi and Lasek, '87), then these observations suggest that primary afferent fibers grow into the grafts prior to the formation of the astroglial barrier at the junction.

Since the analogy between transplants and lamina I is only approximate, it is not surprising that the stereological analysis also demonstrates differences between the composition of the 2 regions. The area fraction occupied by unmyelinated axons is significantly smaller in transplants and that occupied by axon terminals is significantly larger. In part these differences are due to the significantly larger size of terminals in transplants. Other mechanisms may also contribute. For example, reactive synaptogenesis in transplants could increase the area fraction occupied by terminals (Goldberger and Murray, '78, '88; Murray and Goldberger, '86); less branching by unmyelinated axons in transplants compared with lamina I would reduce the area fraction occupied by unmyelinated axons in transplants.

Comparison of regenerated dorsal roots in transplants with dorsal roots in lamina I

Our observation that regenerated dorsal root axons form synapses within transplants allows us to compare the axons and axon terminals derived from a particular group of neurons in transplants and normal spinal cord. The stereological analysis indicates that the area fraction occupied by CGRP-labeled unmyelinated axons in transplants is comparable to that observed in lamina I. However, the area fraction occupied by CGRP-labeled myelinated axons in transplants is decreased by approximately 80%. Because the average diameter of CGRP-labeled myelinated axons in transplants and normal lamina I is virtually the same, this result indicates either that few if any myelinated axons of DRG neuron origin regenerate into transplants or that few CGRP-immunoreactive axons in transplants become myelinated.

The ultrastructure of CGRP-immunoreactive synaptic terminals in transplants suggests that A delta fibers regenerate but remain unmyelinated. In the superficial dorsal horn of normal rats, the terminals of at least some C and A delta fibers are found as the central terminals of synaptic glomeruli. The ultrastructural features and location of these central terminals allow them to be distinguished, and they have been classified as either dark indented CI terminals or

Fig. 10. CGRP-immunoreactive terminals forming axoaxonic synapses within the transplants. Bar = 1 μm. a: CGRP-labeled presynaptic terminal synapses with two different postsynaptic elements (arrows). One is a typical dendrite (D); the other resembles an axon terminal (A), because it contains dense-cored vesicles which are immunoreactive for CGRP. b: CGRP-labeled presynaptic terminal synapses (arrow) with CGRP-labeled axon (A). c: CGRP-unlabeled axon (*) makes a synaptic contact (arrow) on a CGRP-labeled axon (A).

light rounder CII-terminals (Ribeiro-da-Silva and Coimbra, '82; Ribeiro-da-Silva et al., '85). CI terminals are thought to derive from unmyelinated fibers and CII terminals from myelinated fibers (Nagy and Hunt, '81; Ribeiro-da-Silva and Coimbra, '82; Ribeiro-da-Silva et al., '85). The majority of CGRP-immunoreactive complex terminals that we found in both transplants and lamina I resemble CII terminals and are likely to originate from A delta dorsal root fibers. Our observation of numerous labeled complex terminals in transplants therefore suggests that A delta axons grow into transplants but that remyelination of the regenerated axons, if it occurs, takes longer than the 1–3 month period that we studied. Remyelination of regenerated nerves also follows a protracted time course in other systems (Murray, '76).

Most terminals formed by CGRP-containing DRG axons in transplants resemble those in lamina I morphologically, and approximately 75% in the two locations contain both spherical and dense-cored vesicles. The normal proportions of simple and complex terminals are retained in transplants, and the MSI (Raisman, '69; Matthews et al., '76; Hoff et al., '82; Steward and Vinsant, '83) is also unchanged. In addition, we observed that in both transplants and normal spinal cord, 25% of CGRP-labeled terminals are complex. The morphometric analysis, however, shows that terminals immunoreactive for CGRP, like unlabeled terminals, occupy a significantly larger percentage of the area of transplants than of lamina I and that this increase is attributable at least in part to a significant increase in several parameters of terminal size, including area, perimeter, and longest axis. The enlarged terminals occur throughout the transplants and bear specializations indicative of synapses. They therefore differ from the dilated processes that have been reported to end blindly in the dorsal root transitional zone during the early stages of regeneration of dorsal root axons (reviewed in Reier et al., '83a, '89). Their presence in transplants thus represents a successful completion of growth rather than a growth failure. Some of the synapses formed in the superior colliculus by retinal ganglion axons that have regenerated through peripheral nerve bridges are also larger than the normal retinofugal synapses that they otherwise resemble (Carter et al., '88; Bray and Aguayo, '89).

The average length of the synaptic contacts of profiles labeled for CGRP is also significantly larger in transplants than in lamina I. In other areas of the CNS, the length of residual synaptic contacts has been observed to increase following partial denervation, and it is thought to provide a mechanism by which the efficacy of the remaining synapses is enhanced (reviewed in Hillman and Chen, '85). The increased length of synaptic contacts formed by CGRP-containing primary afferent axons in transplants may therefore be a response that permits the regenerating axons to compensate physiologically for the decreased density of synaptic inputs that we also observed in transplants. Decreased synaptic density and increased length of some types of residual synaptic contacts have previously been reported in the dentate molecular layer of hippocampal transplants (Sørensen and Zimmer, '88).

In transplants the majority of both CGRP-containing and unlabeled terminals establish asymmetric synaptic contacts (type I, Gray, '59) on dendrites and therefore resemble synapses in lamina I, approximately 95% of which are axodendritic. In both transplants and lamina I, these dendrites may contain vesicles. The presence of such vesicles in a postsynaptic structure made it difficult to distinguish axodendritic synapses from the axoaxonic synapses that have been identified in a number of different areas of the CNS (reviewed in Peters et al., '76). In most axoaxonic synapses, the postsynaptic terminal also establishes a contact with a dendrite or cell body and its synaptic vesicles are closely associated with the second synaptic interface on dendrite or perikaryon rather than with the axoaxonic interface. We did not observe such a second synaptic complex on dendrite or cell body in transplants or in lamina I, and we classified a synapse as axoaxonic only if the postsynaptic profile was immunoreactive for CGRP. Although this method of identification is likely to cause an underestimation of the number of axoaxonic synapses, the number formed by CGRP-labeled terminals in transplants is significantly greater than we found in lamina I or than has been reported in other areas of normal dorsal horn (Murray and Goldberger, '86).

Our analysis also shows that at least some axoaxonic synapses represent regenerated dorsal root terminals forming synapses with each other. This is clearly so when both synaptic profiles are immunoreactive for CGRP. Because CGRP is present in only about 50% of DRG neurons (Gibson et al., '84), however, we can expect many pre- and postsynaptic profiles in axoaxonic synapses to be unlabeled even though they originate in the dorsal roots. Markers specific for additional subsets of DRG axons (Dodd and Jessell, '85) would presumably show more primary afferent axons establishing axoaxonic synapses with each other.

Several different mechanisms could account for the greater than normal numbers of axosomatic and axoaxonic synapses established in transplants: i) regenerating adult dorsal root axons may differ from growing axons in their ability to detect or respond to the cues directing synapse formation in developing spinal cord; ii) the cues provided by target neurons in transplants may differ from those provided by neurons in the developing dorsal horn; or iii) the coordinated timing between dorsal root ingrowth and spinal cord maturation essential to correct synaptogenesis during development may be disordered. The growth of regenerating dorsal root axons into transplants is delayed compared with growth during normal development, since we have found few CGRP-immunoreactive axons in transplants within 7 days after transplantation of E 14 spinal cord (unpublished observations). During normal development, A delta and C fibers reach the L 4/5 dorsal horn at E 19 (Smith, '83; Fitzgerald, '87; Smith and Frank, '88) and form synapses whose density in laminae I and II has been estimated by light microscopic methods to be complete by birth (Fitzgerald, '87), although not fully functional before the second postnatal week (Fitzgerald and Gibson, '84). In spite of delayed ingrowth, most regenerating DRG axons form synapses in transplants that are similar to those formed in normal spinal cord. Some of the signals that direct synaptogenesis during development therefore appear to be present during regeneration.

The similarities between spinal cord transplants and normal spinal cord demonstrated by the present study encourage the hope that transplants can contribute to at least partial restoration of damaged neuronal circuits. The physiological consequences of such a restoration of anatomical circuitry await future study.

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Regeneration of Adult Dorsal Root Axons Into Transplants of Fetal Spinal Cord and Brain: A Comparison of Growth and Synapse Formation in Appropriate and Inappropriate Targets

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ABSTRACT

Cut dorsal root axons regenerate into transplants of embryonic spinal cord and form synapses that resemble those found in the dorsal horn of normal spinal cord. One aim of the present study was to determine whether these axons also regenerate into and establish synapses within transplants of embryonic brain. A second aim was to compare the patterns of growth in embryonic brain and spinal cord transplants.

Embryonic spinal cord or brain was transplanted into the lumbar enlargement of adult Sprague-Dawley rats, the L4 or L5 dorsal root was cut, and the cut root was juxtaposed to the transplant. The transplants included whole pieces or dissociated cell suspensions of embryonic day 14 (E14) spinal cord, or whole pieces of E14 neocortex, E18 occipital cortex, E15 cerebellum, or E18 hippocampus. One month later the regenerated dorsal root axons were labeled by immunocytochemical methods to demonstrate calcitonin gene-related peptide (CGRP).

CGRP-immunoreactive axons regenerated into all the transplants examined and formed synapses in the neocortex and cerebellum transplants in which they were sought. Synapses were far rarer in neocortex and cerebellum than we had observed previously in transplanted spinal cord, and the patterns of growth differed in transplants of spinal cord and brain. In solid transplants of spinal cord, regenerated axons remained relatively close to the interface with the dorsal root, branched, and formed bundles. Areas of dense ingrowth were separated by regions with few labeled axons. In transplants of brain regions, the regenerated axons were few, unbranched, and appeared as individual fibers rather than in bundles, but they were distributed widely in neocortex transplants. The results of quantitative studies confirmed these observations. The area fraction occupied by regenerated axons in solid spinal cord transplants was significantly larger than in occipital cortex or cerebellum transplants. Distribution histograms of the area occupied in transplants demonstrated that regenerated axons were distributed sparsely but homogeneously in transplants of brain, whereas spinal cord transplants were heterogeneous for regenerated axons and contained areas in which growth was dense or sparse. In contrast, several measurements of axon distribution, including area, longest axis, and length of lateral extension, indicated that CGRP-labeled axons spread more widely in occipital cortex transplants than in solid transplants of spinal cord or cerebellum.

The results indicate that embryonic CNS tissues that are not normal targets support or enhance the growth of severed dorsal roots and suggest that the conditions that constitute a permissive environment for regenerating axons are relatively nonspecific. Embryonic spinal cord, the normal target of dorsal roots, appears to supply additional, more specific cues that enable regenerating axons to grow and arborize within the transplant and to establish relatively normal numbers of synapses. These cues appear to depend at least in part on the integrity of transplant structure, since growth into solid transplants of spinal cord exceeds growth into cell suspensions.

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CGRP immunocytochemistry

One month after transplantation the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Spinal segments containing transplants were removed, sectioned in the sagittal plane on a cryostat (10 μm), and mounted on subbed slides. To identify regenerated axons, every fifth section was processed for CGRP immunocytochemistry according to the avidin-biotin method of Hsu et al. ('81). Sections were reacted with primary antiserum against CGRP (Peninsula Laboratories, Belmont, CA) at 1:8,000 dilution, and then immersed in biotinylated goat antirabbit IgG and avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) as specified by the manufacturer. The chromagen was 3,3'-diaminobenzidine (DAB). The characteristics of the primary antisera against CGRP and controls for specificity of staining have been described previously (Tessler et al., '88).

To evaluate transplant morphology and the dorsal root-transplant and transplant-host spinal cord interfaces, additional sections were stained with chromoxane cyanine R for myelin and counterstained with cresyl violet (Clark, '81). To determine the localization of regrowing axons within the transplants, selected sections were stained with CGRP immunocytochemistry and counterstained with cresyl violet.

To determine whether regenerated dorsal root axons formed synapses with neurons in brain transplants, occipital pole transplants of E14 neocortex (n = 2) and E15 cerebellum transplants (n = 3) were prepared for electron microscopic analysis. Immunocytochemical procedures were identical to those that we used previously to study E14 spinal cord transplants (Itoh and Tessler, '90) except that in the present analysis we substituted the ABC procedure for the peroxidase-antiperoxidase method. One month postoperatively the rats were deeply anesthetized with sodium pentobarbital and perfused with 500 ml of a fixative containing 3% glutaraldehyde, 3% paraformaldehyde, and 0.1% picric acid in 0.1 M cacodylate buffer at pH 7.4. Segments 10 mm long that contained transplants were immediately removed. Sections were cut with a Vibratome in the sagittal plane at 50 μm and treated with 1% sodium borohydride in phosphate-buffered saline (Kosaka et al., '86). After overnight incubation in anti-CGRP antiserum at 1:16,000 dilution, the sections were processed for the ABC procedure by using DAB as chromagen. The sections were then osmicated (Ruda et al., '86), stained en bloc in 1% aqueous uranyl acetate, dehydrated, and flat-embedded in Epon-Araldite mixture. Ultrathin sections were cut from regions within 1 mm of the dorsal root-transplant interface that contained CGRP-labeled axons, placed on thin-meshed 200 grids, and examined in a JEOL 100S electron microscope.

Quantitative analysis

The extent to which CGRP-labeled axons regenerated into transplants was measured for whole pieces of E14 spinal cord (N = 5), E18 occipital cortex (N = 5), and E15 cerebellum (N = 5), and for dissociated cell suspensions of E14 spinal cord (N = 5). In these transplants regenerated axons entering directly from the cut dorsal roots could be distinguished with certainty from dorsal root collaterals that entered the transplants after ascending or descending in the host spinal cord (Houle and Reier, '89; Traub et al., '89); staining deriving from the two sources was separated by an area in which CGRP immunoreactivity was scanty or absent. E14 neocortex transplants were not analyzed quantitatively because the two types of regenerated dorsal root axons occasionally intermingled.

To determine the extent of CGRP-immunoreactive axon regeneration within each transplant, we used a point-counting stereological analysis carried out with a Bioquant System IV (R & M Biometrics, Inc., Nashville, TN) to measure the area occupied by labeled axons. For the stereological evaluation, sagittal sections that contained CGRP-immunoreactive axons were examined under a light microscope (×40) and projected onto a video monitor at a final magnification ×1,086. An 180 mm × 180 mm sampling lattice (2.62×10^4 μm^2) composed of 10-mm squares was superimposed over the video monitor, and the number of times that CGRP-labeled axons intersected the corners of the grid squares was counted. Five consecutive sections at ten section intervals were examined per transplant, with the third of these five sections containing the most abundant CGRP immunoreactivity in that transplant. The area occupied by labeled axons in each individual sampling lattice (2.62×10^4 μm^2) was also calculated, and a distribution histogram was made from these data to show regional variations within the transplant of the area occupied by these axons.

To determine the arborization of CGRP-immunoreactive axons within the three dimensions of each transplant, we measured both the distribution of labeled axons in the sagittal plane and their lateral extension. The distribution of CGRP-immunoreactive axons in the sagittal plane was determined by making composite montages that consisted of all the individual sampling lattices examined. The longest dimension of the area of the regenerated axons was measured by using the Bioquant System IV. The lateral extension of CGRP-immunopositive axons was calculated by multiplying the number of sections that contained CGRP-labeled axons by the section interval (50 μm). We also estimated the overall volume of these transplants (1 month after transplantation). Every tenth section cut in the sagittal plane was stained with chromoxane cyanine R and cresyl violet. The area of these sections was measured with the Bioquant IV, and the sum of the areas was multiplied by the distance between sections (100 μm). The initial volume of the transplants was determined by estimating the volume contained in the Pasteur pipette (1 mm inside diameter) used to introduce the transplant into the lesion cavity.

Because the number of dorsal root axons that were juxtaposed to the transplants might vary among the recipients, we determined the size of the cut dorsal root in each animal. We measured the length of the contact between cut dorsal root and transplant at the dorsal root-transplant interface and the diameter of the dorsal root 750 μm from the interface. We also determined the width of the dorsal root that was apposed to each transplant by multiplying the number of sections that contained the dorsal root-transplant apposition by the section thickness.

The significance of the quantitative comparisons among transplant groups was evaluated by one-way ANOVA. If there were significant ($P < 0.05$) differences among the types of transplants, each result was corrected for multiple comparisons by using Duncan's Multiple Range Test. Statis-

Key words: CNS regeneration, calcitonin gene-related peptide, immunocytochemistry, electron microscopy

The cut central processes of adult mammalian dorsal root ganglion (DRG) neurons regenerate within the dorsal root but do not penetrate the spinal cord (Reier et al., '83a,b, '89; Liuzzi and Lasek, '87). When normal spinal cord is replaced by a transplant of embryonic spinal cord, cut dorsal root axons cross the interface between host dorsal root and transplant, grow within the transplant, and establish synapses that resemble those formed in the normal dorsal horn (Tessler et al., '88; Itoh and Tessler, '90). The normal or homotypic target of the dorsal roots therefore supports or enhances growth of the cut axons and provides cues necessary for synapse formation.

It is not known whether the properties of the transplant that enable regenerating axons to grow and establish synapses are specific to the normal target, or if they are common to embryonic CNS tissue. The early outgrowth of developing axons is thought to depend on signals such as extracellular matrix and cell-cell adhesion molecules that are expressed generally throughout the embryonic CNS (reviewed in Jessell, '88). Regenerating axons may have more specific requirements for growth, however, because both cut embryonic DRG neurites (Smalheiser et al., '81) and cut young adult retinal ganglion cell axons (Harvey et al., '87) grow only sparsely into inappropriate or heterotypic embryonic targets. The signals that allow developing axons to grow within a target (Keller et al., '89) and establish synapses (reviewed in Jessell, '88) appear to be more specific than those that direct initial axon extension. The requirements of adult regenerating axons may again differ from those of developing axons, however, because the cut adult retinal ganglion cell axons that have grown through a peripheral nerve graft form synapses with neurons of the adult cerebellum (Zwimpfer et al., '89).

The present study investigates whether regenerating adult dorsal root axons can grow into and form synapses in transplants of tissues that are not their normal targets and whether the patterns of growth and synapse formation differ in homotypic and heterotypic targets. We attempt to distinguish characteristics of growth that are target-specific from those shared by non-target embryonic CNS tissue, and thus to begin to define the contributions of specific and nonspecific mechanisms. We use quantitative morphological methods to compare the pattern of regenerated dorsal root axon growth and synapse formation in transplants of embryonic spinal cord with the patterns found in inappropriate targets provided by transplants of several different embryonic brain regions. We use immunocytochemical methods for demonstrating regenerated axons immunoreactive for calcitonin gene-related peptide (CGRP), and study growth into regions of brain that either normally receive (hippocampus, cerebellum) or lack (occipital cortex) CGRP innervation. Because cell suspensions as well as whole pieces of embryonic spinal cord have been used to promote spinal cord repair (Houle and Reier, '88), we also examine the effects on growth of disrupting transplant structure; we compare the pattern of growth into transplants of whole pieces of embryonic spinal cord with the pattern seen in transplants of dissociated spinal cord.

MATERIALS AND METHODS

Transplants from 53 rats were studied. The transplants were whole pieces (n = 16) or dissociated cell suspensions (n = 7) of embryonic day 14 (E14) rat spinal cord, or whole pieces of E14 neocortex (n = 7), E18 occipital cortex (n = 10), E15 cerebellum (n = 9), or E18 hippocampus (n = 4). Table 1 summarizes the analyses performed.

Surgical procedures

Female adult (200–350 g) Sprague-Dawley rats received transplants. The rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (95 mg/kg), xylazine (10 mg/kg), and acepromazine maleate (0.7 mg/kg), and a laminectomy of the T13 or L1 vertebra was performed with a speed drill (Dremel, Racine, WI). After the left L4 or L5 dorsal root was sharply transected close to the dorsal root entry zone and reflected caudally, a hemisection cavity 3 mm in length or a dorsal funiculotomy cavity 2 mm in length was produced in the lumbar enlargement by gentle subpial aspiration. Segments of spinal cord or brain were dissected from embryos of the appropriate age and introduced into the cavity according to procedures previously described (Reier et al., '86). Dissociated cell suspensions of E14 spinal cord were prepared by triturating entire spinal cords through a graded series of hypodermic needles followed by centrifugation (Houle and Reier, '89). The severed dorsal root stump was juxtaposed to the caudal one-third of the dorsal surface of the transplant, the resected vertebral arch was replaced, and the superficial wound was closed in layers. This procedure has been described in detail (Reier et al., '86; Tessler et al., '88; Itoh and Tessler, '90).

TABLE 1. Embryonic CNS Grafts (Mean ± S.E.M.)

Group	N	Quantitative analysis[1]	EM[2]	Lesion	Tissue	Final graft volume (mm^3)	Increase of graft volume[3] (%)
E14 spinal cord	16	5	—	HSC[4]	Solid	3.74 ± 0.82	156.80 ± 34.86
E14 spinal cord	7	5	—	HSC	DCS[5]	3.78 ± 0.22	160.61 ± 9.54
E14 neocortex	7	—	2	HSC	Solid	—	—
E18 occipital cortex	10	5	—	HSC	Solid	4.70 ± 0.72	199.48 ± 30.73
E15 cerebellum	9	5	3	DQC[6]	Solid	1.70 ± 0.46	143.99 ± 39.24
E18 hippocampus	4	—	—	DQC	Solid	—	—

[1]N of animals for quantitative analysis.
[2]N of animals for electron microscopic analysis.
[3]There are not significant differences among groups ($P > 0.05$).
[4]HSC: hemisection cavity
[5]DQC: dorsal quadrant cavity.
[6]DCS: dissociated cell suspensions.

tical significance of graft volume increase and histogram of area occupied by CGRP-labeled axons was determined by the Kruskal-Wallis one-way ANOVA. If significant differences were present ($P < 0.05$), individual posthoc comparisons were corrected with the Wilcoxon-Mann-Whitney test for multiple comparisons. All statistical analyses were performed by using the Number Cruncher Statistical System (Dr. Jerry L. Hintze, Kaysville, UT).

RESULTS

General histology

Spinal cord and brain transplants survive in the adult host spinal cord and differentiate into patterns that are characteristic for each CNS region. The patterns found in our material stained with chromoxane cyanine R and cresyl violet are similar to those previously described for transplants taken from these regions (spinal cord: Bernstein et al., '84; Reier et al., '86; Tessler et al., '88; Jakeman et al., '89; Itoh and Tessler, '90; neocortex: Jaeger and Lund, '80; Patel and Bernstein, '83; hippocampus: Kromer et al., '83; cerebellum: Wells and McAllister, '82; Kromer et al., '83; Takayama et al., '87). All of these transplants contain the neuronal and non-neuronal elements that comprise the normal neuropil as well as both myelinated and unmyelinated areas (Figs. 1–5).

Transplants of solid pieces and dissociated cell suspensions of spinal cord and neocortex transplants lack the overall architecture and the unequivocal lamination patterns of neurons found in normal mature spinal cord and neocortex (Figs. 1–3). Spinal cord transplants, however, contain regions that resemble substantia gelatinosa based on the presence of numerous small neurons and relative paucity of myelination (Fig. 1c) (Reier et al., '83, '85, '86; Jakeman et al., '89). Neocortex transplants include areas in which cellular bands composed of small- or medium-sized neurons resemble normal cortical layers (Fig. 3c). Hippocampus transplants contain regions that resemble subdivisions of the normal adult hippocampus (Kromer et al., '83) because medium-sized cells are organized into cell aggregates that are separated by regions of neuropil (Fig. 5b). In most of our hippocampus transplants granule cells form aggregates at the dorsal root-transplant interface. Cerebellum transplants most closely resemble the organization of the normal mature tissue. All develop a well-organized trilaminar pattern consisting of granule cell, Purkinje cell, and molecular layers (Fig. 4c), but the polarity of the normal trilaminar pattern is reversed. Purkinje cells are occasionally found in the molecular layer of transplants, and lobulations consisting of internal and external granule cell layers surrounding a Purkinje cell layer are frequent.

Transplants are generally well-integrated with host spinal cord and host dorsal root (Figs. 1a, 2a, 3a,b, 4a,b, 5a). The interface between the regenerated host dorsal root and transplant, however, is readily recognized by the clear contrast in cell density between the numerous closely packed glial cells found in the nerve roots and the more loosely cellular transplants.

Transplants of spinal cord and brain grow within the adult host spinal cord. After 1-month survival the volume of solid tissue spinal cord transplants increases by 160% over the initial volume, dissociated cell suspensions by 160%, occipital cortex by 200%, and cerebellum by 140%. The increases shown by these transplants are not significantly different from each other (Table 1).

CGRP immunocytochemistry

Dorsal root axons immunoreactive for CGRP regenerate into every transplant examined (Figs. 6–11). Most of these labeled axons can be observed to course longitudinally within dorsal roots apposed to the transplants and then to extend into the grafts. Regenerated axons within every transplant show varicosities, as do CGRP-containing dorsal root axons in normal dorsal horn (Traub et al., '89), but the varicosities are more common in spinal cord transplants than in brain grafts. CGRP-labeled axons show distinctive patterns of distribution within the transplants of the four different CNS regions.

Spinal cord. CGRP-labeled axons arborize extensively near the surface of transplants of whole pieces of spinal cord and in some portions the axons form dense bundles (Fig. 6) (see also Tessler et al., '88; Itoh and Tessler, '90). CGRP-immunoreactive axons also grow extensively within dissociated cell suspensions of spinal cord, but bundles of CGRP-labeled axons are infrequent (Fig. 7).

Cerebral cortex. CGRP-immunoreactive axons are distributed similarly in E14 neocortex (Fig. 8) and E18 occipital cortex (Fig. 9) transplants. In contrast to the pattern seen in spinal cord transplants, CGRP-labeled axons extend sparsely but diffusely through the neocortex transplants, and individual axons but not bundles of axons can be recognized. Axons regrowing into E14 neocortex directly from the dorsal root occasionally intermingle with ascending or descending primary afferent collateral axons entering from host spinal cord (Fig. 8a).

Cerebellum. Regenerated CGRP-labeled axons extend into each of the three layers found in transplants of E15 cerebellum (Fig. 4). The axons form relatively dense bundles in the granule cell and molecular layers (Fig. 10), but few extend into the Purkinje cell layer, and these occur as individual fibers rather than in bundles. The arborization of CGRP-labeled axons within cerebellum grafts is distinctly poorer than in spinal cord and neocortex transplants.

Hippocampus. A small number of CGRP-immunoreactive axons extend widely within these grafts, where they form neither dense plexuses nor bundles (Fig. 11). The axons do not distribute to particular locations within the grafts and the limited extent of the growth is similar to that found in cerebellar implants.

Quantitative analysis

The point-counting stereological analysis shows the area fraction of the four types of CNS transplants that is occupied by regenerated dorsal root axons labeled for CGRP (Table 2). In solid spinal cord transplants these axons occupy a mean area of $5.63 \times 10^4\ \mu m^2$. In dissociated cell suspensions of spinal cord the area occupied is approximately 71% of that in solid grafts of spinal cord, in occipital cortex grafts 53%, and cerebellar transplants 18%. Regenerated CGRP-immunoreactive axons therefore occupy a significantly larger area of solid spinal cord grafts than of either type of brain graft or of dissociated cell suspensions prepared from E14 spinal cord.

Distribution histograms of the area occupied by labeled axons per individual sampling lattice ($2.6 \times 10^4\ \mu m^2$) show further differences in the pattern of growth in the various transplants (Fig. 12). The mean percentage of sampling lattices in which CGRP-labeled axons occupy less than $1{,}000\ \mu m^2$ is nearly 40% in solid spinal cord transplants, but

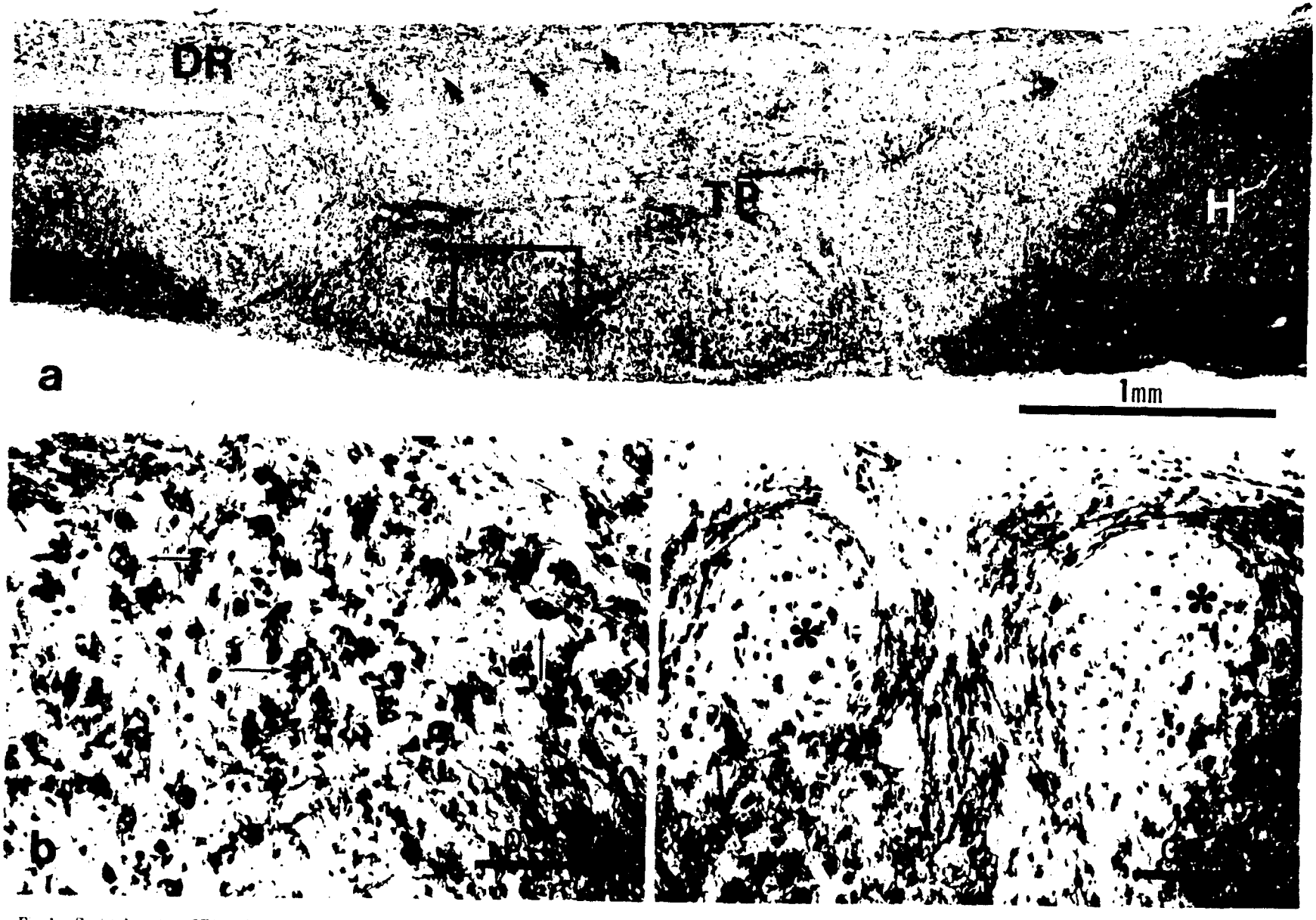

Fig 1 Sagittal section of E14 spinal cord solid tissue graft 1 month after transplantation **a**: Transplant (TP) is integrated with host spinal cord (H) and dorsal root (DR) (arrows) Transplant does not show the laminar structure characteristic of normal spinal cord **b**: A more highly magnified photograph of the area indicated by the rectangle in Fig 1a shows medium-sized neurons (arrows) in a central portion of the transplant **c**: Myelin free regions (*) surrounded by myelinated fibers consist of aggregates of small neurons Stained with chromoxane cyanine R and cresyl violet

Fig. 2. Sagittal section of E14 spinal cord dissociated cell suspension graft 1 month after transplantation. **a**: Transplant (TP) fused with host dorsal root (DR) (arrows) and spinal cord (H) contains neurons of variable size. **b**: A more highly magnified photograph of the area (b) outlined by a rectangle in Figure 2a shows large neurons (arrows). **c**: Detail of the area outlined by a rectangle (c) in Figure 2a shows a central canal like structure characteristic of dissociated cell suspension transplants. The densely packed small cells resemble ependymal cells. Stained with chromoxane cyanine R and cresyl violet

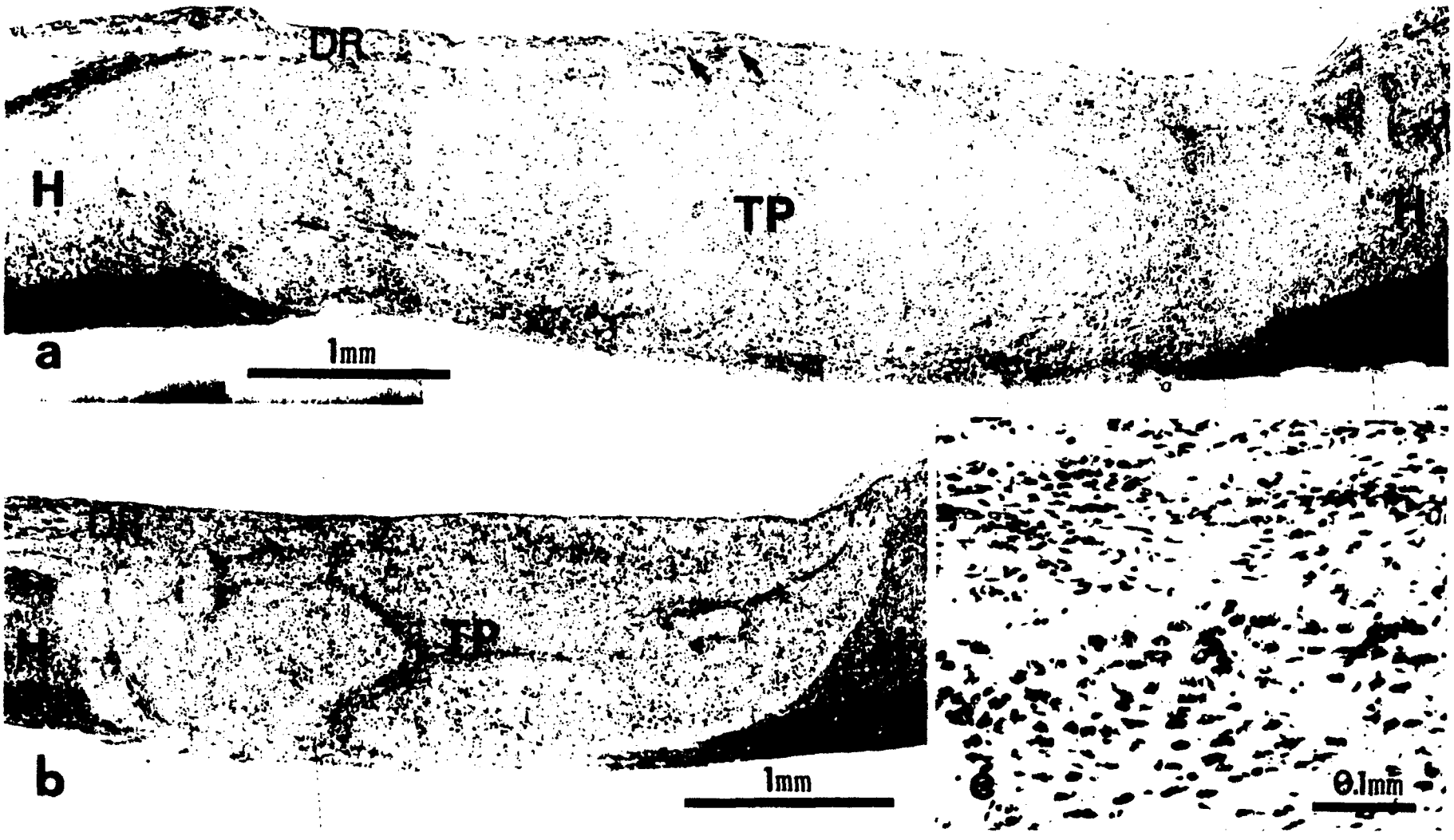

Fig. 3. Sagittal sections of neocortex grafts 1 month after transplantation. a: E14 neocortex transplant (TP) is integrated with rostral and caudal host spinal cord (H) and dorsal root (DR) (arrows). The laminar pattern characteristic of normal cerebral cortex is not seen. b: E18 occipital cortex transplant (TP) also fuses with host spinal cord (H) and dorsal root (DR) (arrows). c: A more highly magnified photograph of another E14 neocortex transplant shows cellular bands consisting of medium sized neurons. Stained with chromoxane cyanine R and cresyl violet.

Fig. 4. Sagittal section of E15 cerebellum graft 1 month after transplantation. a: Transplant (TP) is integrated with host dorsal root (DR) and spinal cord (H). b: A higher-magnification photograph of the region indicated by the rectangle in Figure 4a shows the interface (arrows) between host dorsal root (DR) and transplant (TP). c: An example of the trilaminar structure within a different cerebellum transplant consisting of granule cell (G), Purkinje cell (P), and molecular (M) layers. Stained with chromoxane cyanine R and cresyl violet.

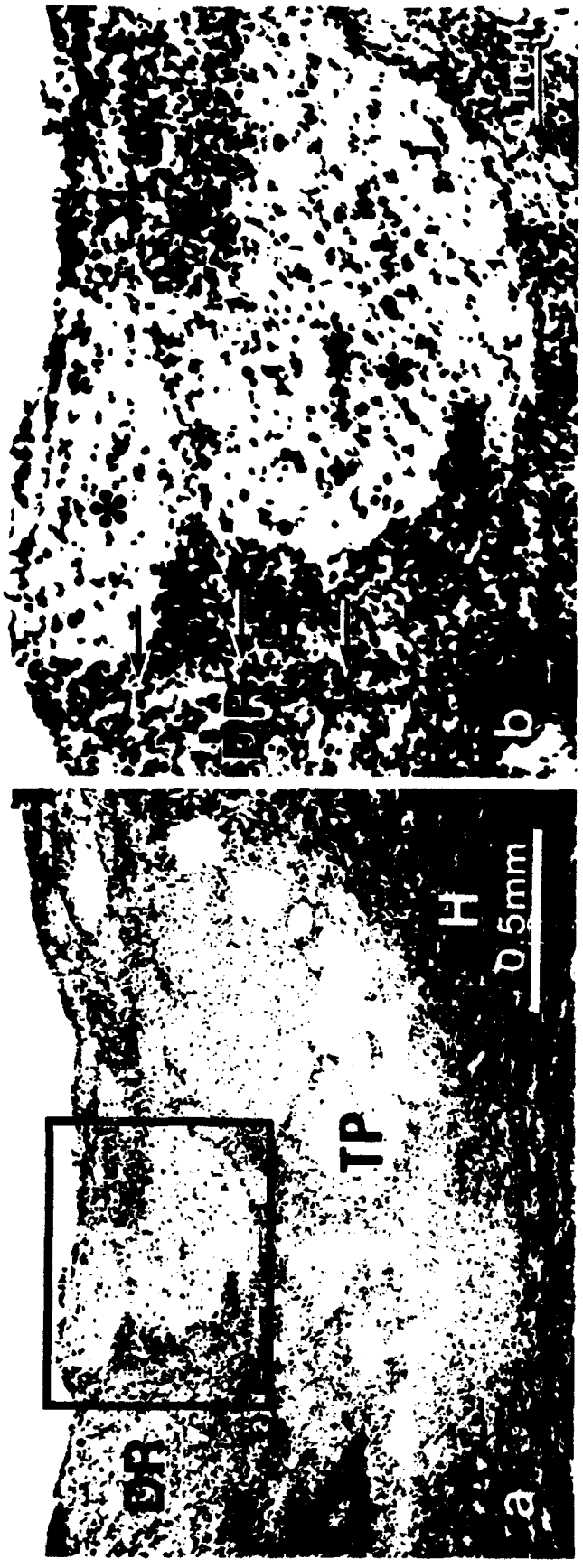

Fig. 5. Sagittal section of E18 hippocampus graft 1 month after transplantation. a: Transplant (TP) integrates with host spinal cord (H) and dorsal root (DR). b: An enlargement of the region indicated by the rectangle in Figure 5a shows cellular aggregates (*) composed of small pyramidal neurons surrounded by dense granule cells. Stained with chromoxane cyanine R and cresyl violet.

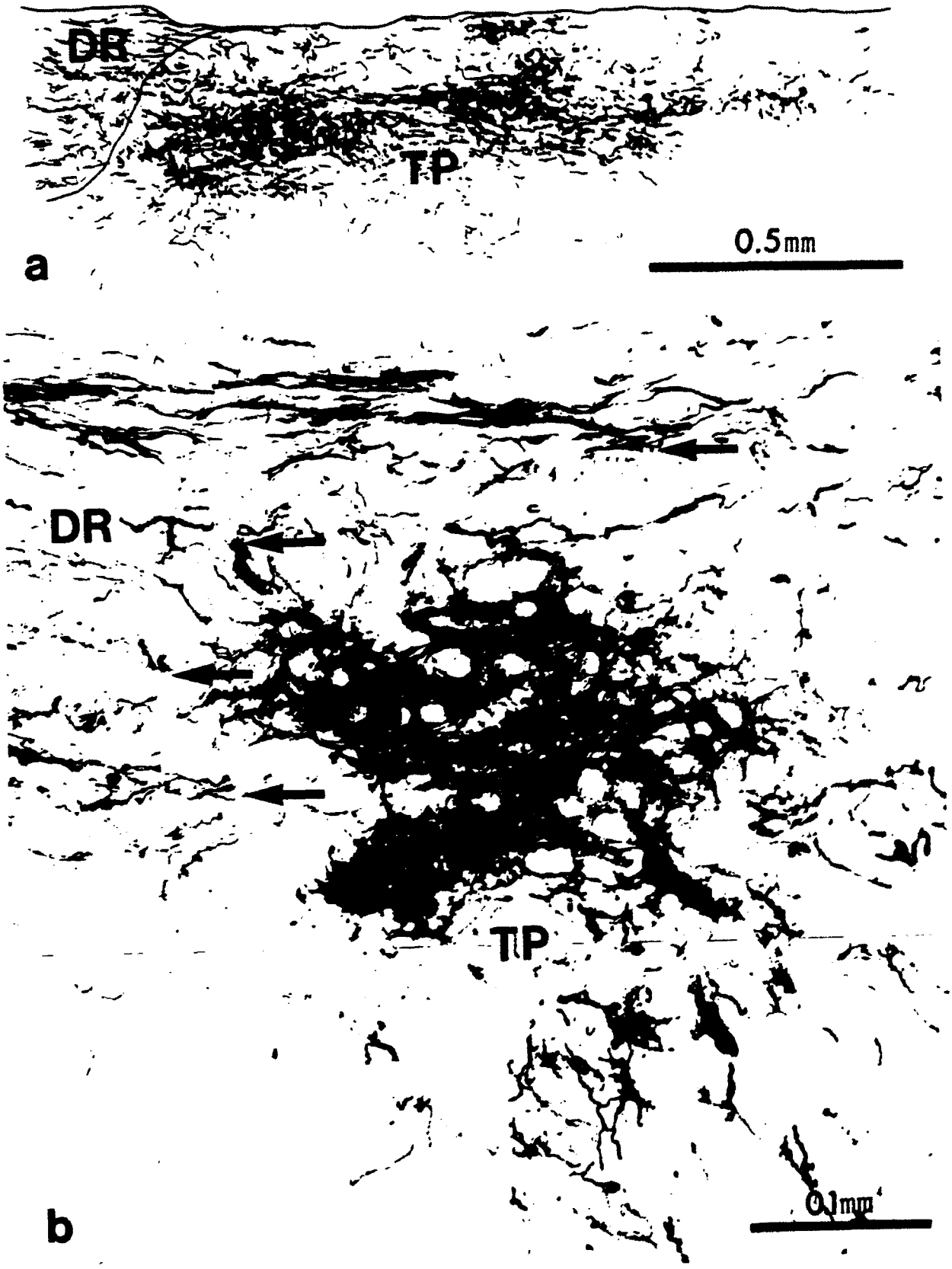

Fig. 6. CGRP-immunoreactive axons in E14 spinal cord solid tissue grafts 1 month after transplantation. Sagittal sections. a: A camera lucida drawing shows rich regrowth of CGRP-labeled axons derived from host dorsal root (DR) in transplant (TP). b: Regenerated axons cross the interface (arrows) between host dorsal root (DR) and transplant (TP) and form dense plexus near the interface. Interface was identified in the adjacent Nissl-stained section.

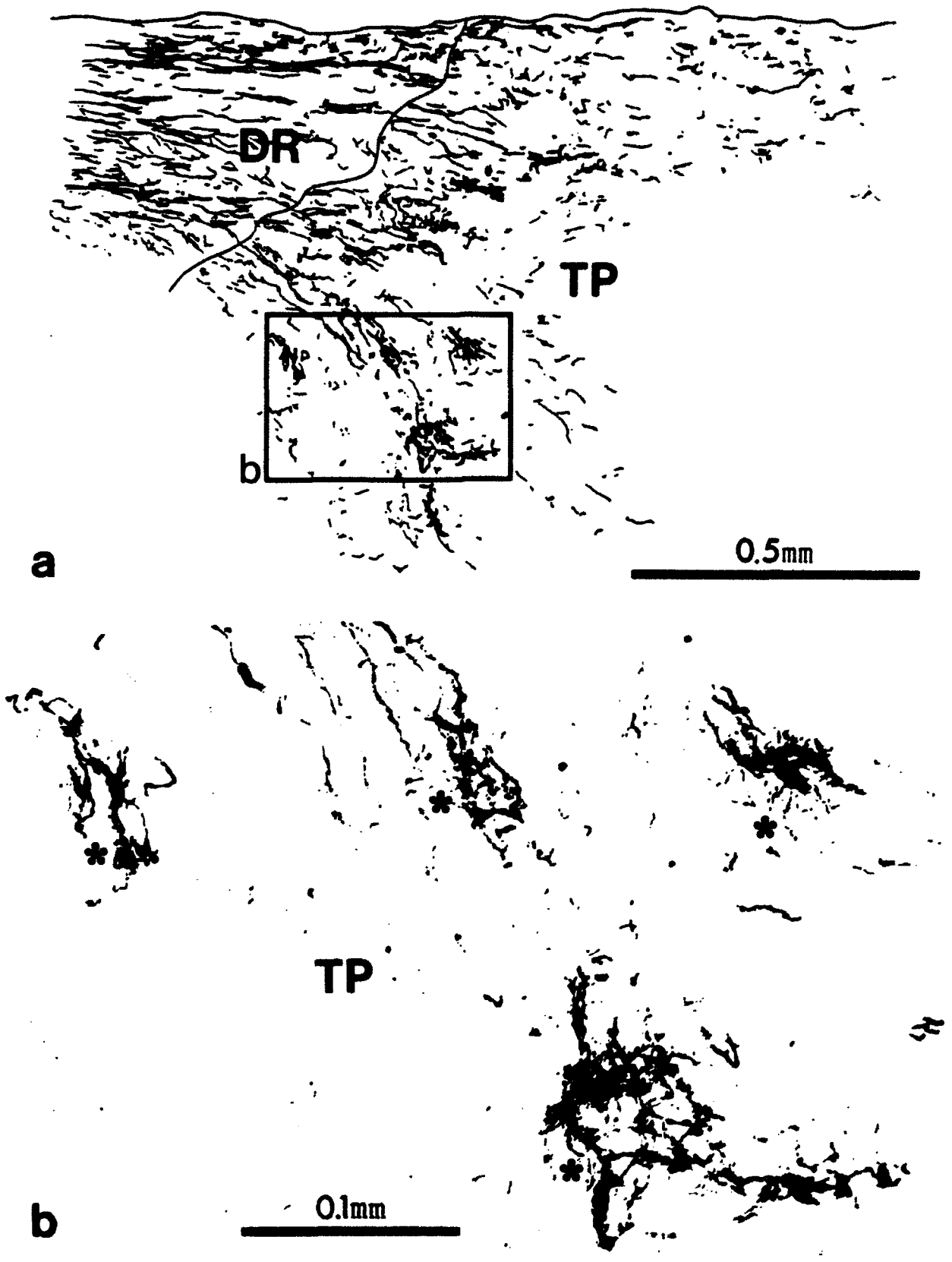

Fig. 7. CGRP-immunoreactive axons in E14 spinal cord dissociated cell suspension graft 1 month after transplantation. Sagittal sections. a: A camera lucida drawing shows axons in host dorsal root (DR) crossing the DR-transplant (TP) interface and distributing within the transplant. b: A more highly magnified photograph of the area outlined by the rectangle in Figure 7a shows relatively dense plexuses (*) of regenerated axons.

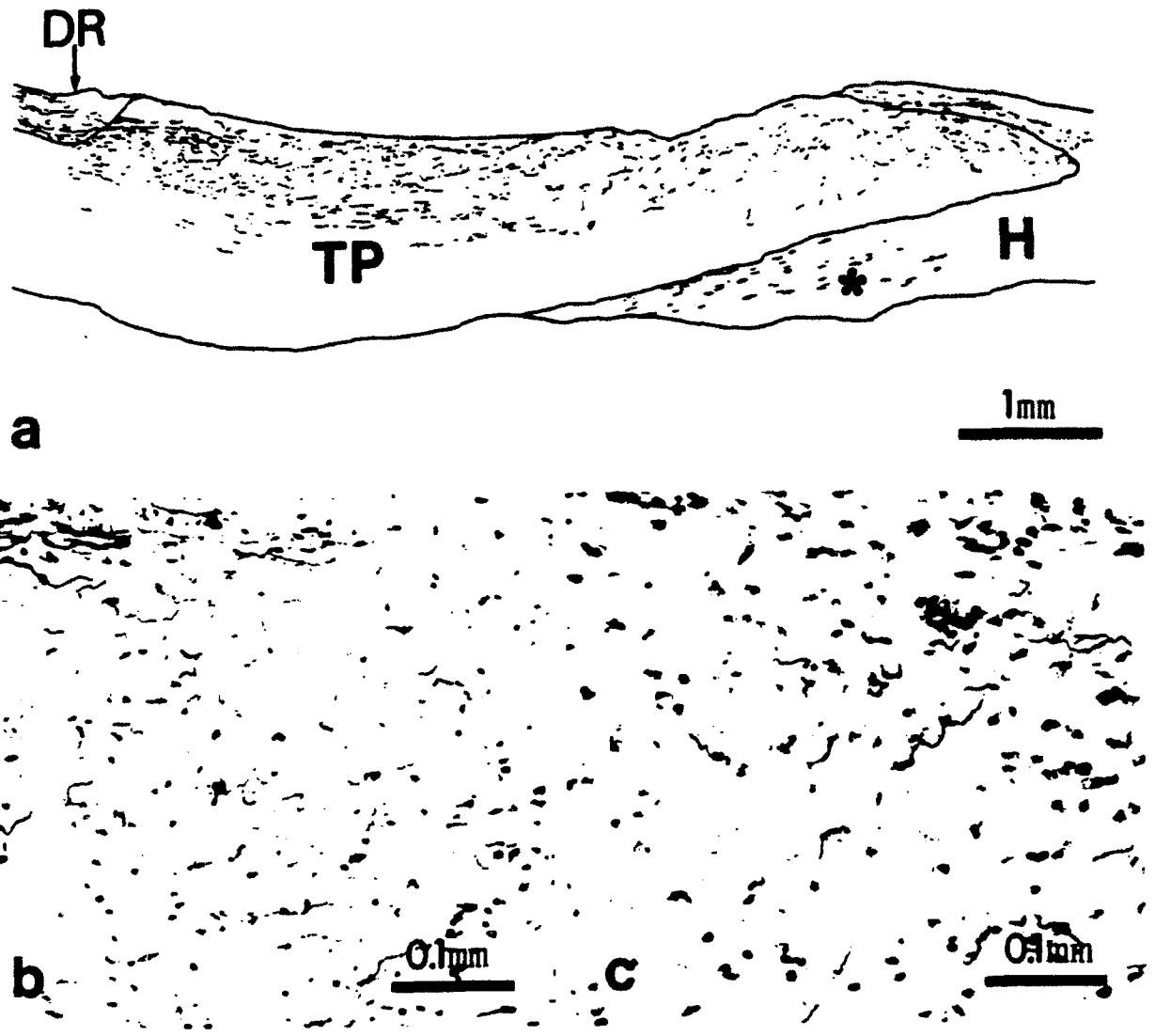

Fig. 8. CGRP-immunoreactive axons in E14 neocortex graft 1 month after transplantation. Sagittal sections. a: Primary afferent axons traverse dorsal root (DR)-transplant (TP) interface, extend into transplant, and intermingle with CGRP-labeled descending axons in host spinal cord (H). CGRP-immunoreactive neurons in host anterior horn are stained (*). b: Higher-magnification photograph of a region near the dorsal root-transplant interface shows that regenerated axons distribute diffusely within the transplant but do not form dense plexuses. Stained with CGRP immunocytochemistry and cresyl violet. c: Higher-magnification photograph of a region near the interface between transplant and rostral host spinal cord. It cannot be determined whether labeled axons derive from the dorsal root or from axons descending from rostral host spinal cord. Stained with CGRP immunocytochemistry and cresyl violet.

60% in dissociated cell suspensions of spinal cord, 90% in neocortex transplants, and 80% in cerebellar transplants. In addition, no sampling lattices are found in which regenerated axons occupy more than 4,000 μm^2 in neocortex or cerebellum transplants, whereas solid spinal cord transplants contain sampling lattices in which regenerated axons occupy areas greater than 8,000 μm^2. The distribution histogram for dissociated cell suspensions of spinal cord shows a pattern of growth intermediate between that of the solid spinal cord transplants and that of the occipital cortex or cerebellum transplants. These results confirm our qualitative observations that the regenerated CGRP-labeled axons show different patterns of growth within the various transplants. Growth into spinal cord transplants is heterogeneous; some spinal cord regions contain sparse growth; in other regions growth is dense. Growth into occipital cortex and cerebellum transplants is relatively uniform but sparse.

Table 3 shows the areas over which regenerated CGRP-labeled axons arborize within the four types of transplants. The mean area of CGRP-innervated regions within occipital cortex transplants is approximately three times that in solid spinal cord transplants and more than four times that in cerebellum implants. The regenerated dorsal root axons therefore distribute over a significantly larger area in occipital cortex grafts than in spinal cord or cerebellum transplants. The longest dimension of each area as well as the length of the lateral extension of the labeled axons is also significantly greater in occipital cortex grafts than in solid spinal cord or cerebellum transplants (Table 3). These results indicate the widespread distribution of CGRP-

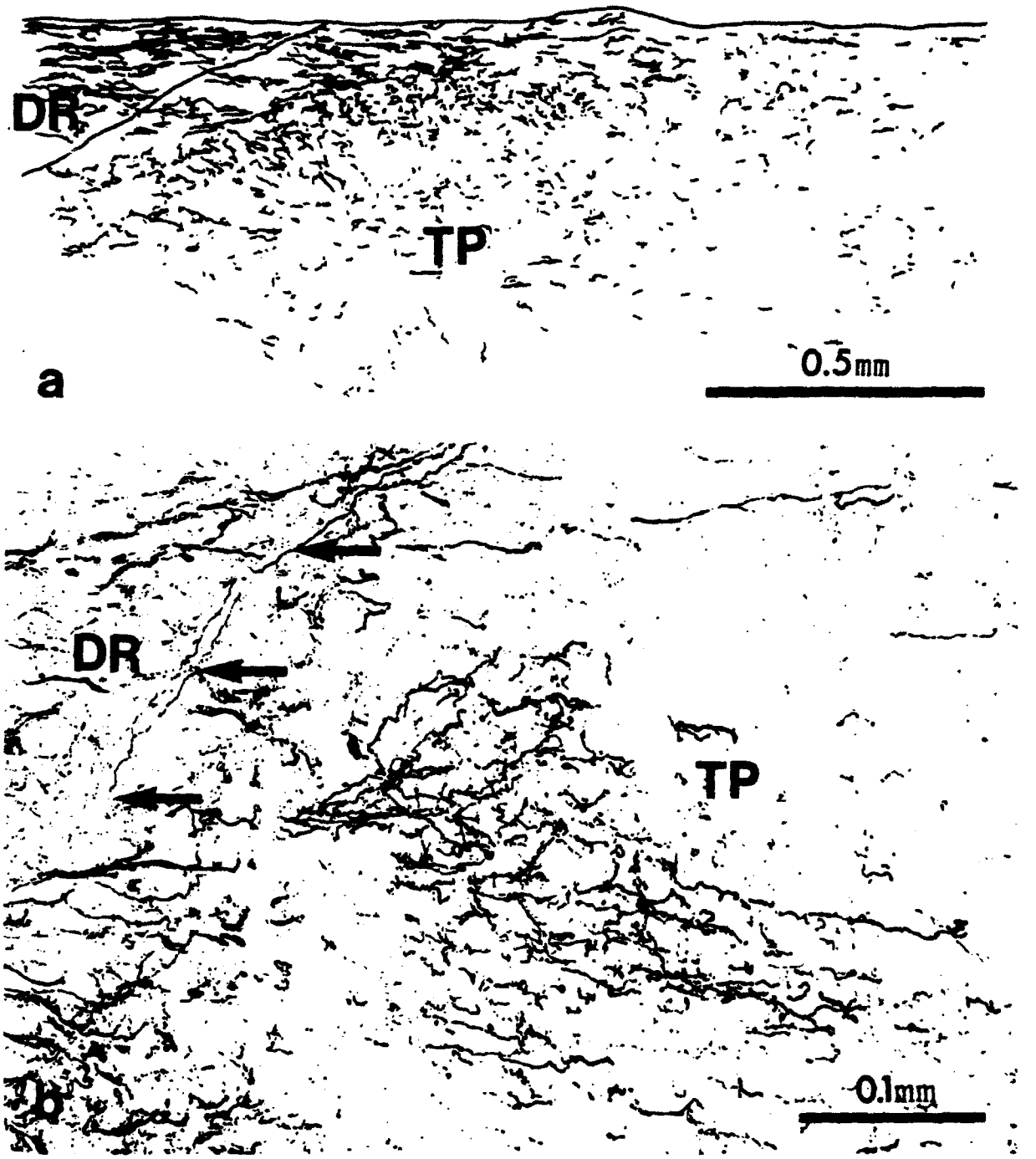

Fig. 9. CGRP-immunoreactive axons in E18 occipital cortex graft 1 month after transplantation. Sagittal sections. a: Camera lucida drawing shows that regenerated axons distribute extensively but diffusely within the transplant (TP), similar to the pattern observed in E14 neocortex transplants. b: Labeled axons cross the dorsal root (DR)-transplant (TP) interface (arrows) and grow within the transplant without the formation of obvious plexuses.

labeled fibers within occipital cortex transplants. The lateral extension of labeled axons within dissociated cell suspensions of spinal cord is also significantly greater than in solid spinal cord grafts, indicating more diffuse growth in transplants of dissociated cell suspensions (Table 3).

The contact length between the cut dorsal root and transplant at the dorsal root-transplant interface, the diameter of the dorsal root 750 μm from the interface, and the width of the dorsal root-transplant apposition are not significantly different among the four groups of transplants

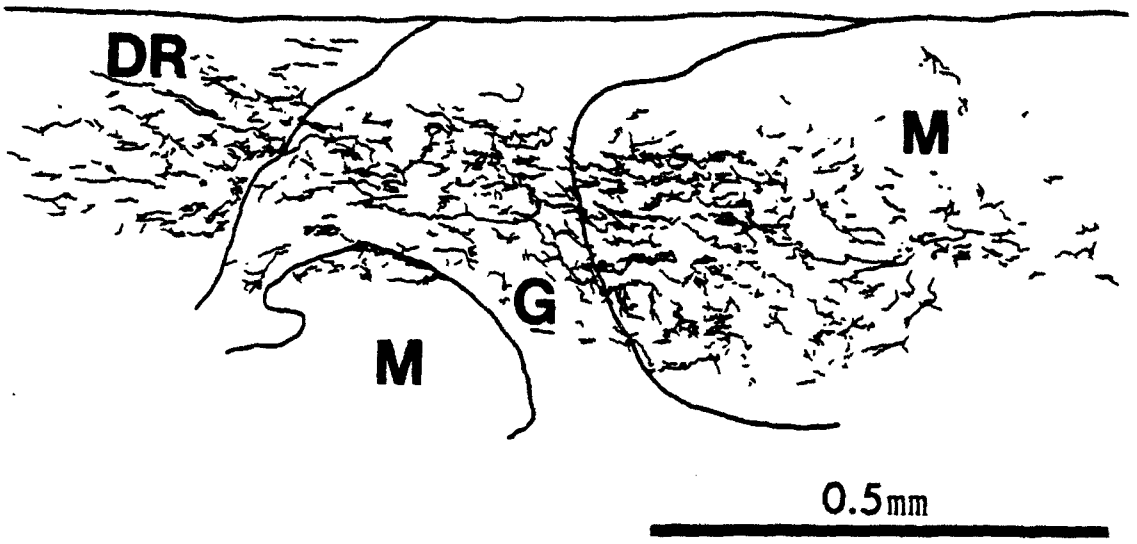

Fig. 10. CGRP-immunoreactive axons in E15 cerebellum graft 1 month after implantation. Sagittal section. Camera lucida drawing shows diffuse and extensive regeneration into molecular (M) and granule cell (G) layers.

(Table 4). We have not counted CGRP-labeled dorsal root axons at the time that they were severed and after regrowth and therefore cannot determine whether innervation of the transplants is due to sprouting of additional DRG axons as well as regrowth of those that were initially cut. The present results, however, suggest that differences in the numbers of dorsal root axons juxtaposed to the transplants do not account for the differences in the extent of regeneration that we have observed.

Electron microscopy

Regenerated CGRP-immunoreactive axons form synapses in neocortex (Fig. 13, 15a) and cerebellum transplants (Figs. 14, 15b). Labeled axons and axon terminals are rare, however, because we seldom find more than one or two labeled profiles per grid square even in CGRP-innervated regions. As in normal dorsal horn (Carlton et al., '88; McNeill et al., '88; Itoh and Tessler, '90) and spinal cord transplants (Itoh and Tessler, '90), CGRP labeling is largely associated with microtubules in axons and with the cores of dense-cored vesicles in axon terminals. Most CGRP-immunoreactive terminals contain two types of synaptic vesicles: a larger number of small, clear, spherical vesicles (mean 50 μm in diameter) and a smaller number of large, spherical, dense-cored vesicles (mean 95 μm in diameter). In contrast to previous studies which used peroxidase-antiperoxidase methods (Carlton et al., '87, '88; McNeill et al., '88; Itoh and Tessler, '90), we find that the axolemma and spherical vesicles are rarely labeled with the ABC method.

Most CGRP-labeled profiles in both cerebral cortex and cerebellum transplants form compound structures consisting of two or three axon terminals rather than the individual profiles found in normal superficial dorsal horn (McNeill et al., '88; Itoh and Tessler, '90). These results are similar to our previous observations of CGRP-labeled axons in spinal cord transplants (Itoh and Tessler, '90). Most CGRP-labeled synaptic complexes in both cerebral cortex and cerebellum transplants are asymmetric (type 1, Gray, '59), and the postsynaptic structure is a dendrite with or without vesicles. Most contact one postsynaptic profile (Figs. 13a, 14a,c), but some form complex synapses with two or more postsynaptic profiles (Figs. 13b, 14b,d). CGRP-labeled axon terminals are not observed to make axosomatic synapses but do contact perikarya by puncta adherentia (Fig. 15a,b). CGRP-labeled synaptic terminals are present in the granule cell (Fig. 14a,b) and molecular (Fig. 14c,d) layers of cerebellum transplants. Some of the labeled terminals found in the granule cell layers are in clusters consisting of two to three axon terminals connected by puncta adherentia, consistent with the light microscopic findings of axon bundles in the granule cell layer.

Table 5 summarizes the morphometric analysis of CGRP-labeled synaptic terminals found in neocortex and cerebellum transplants. The area, perimeter, and the longest dimension of CGRP-labeled synaptic terminals in neocortex transplants appear to be larger than those found in spinal cord transplants and normal superficial dorsal horn (Itoh and Tessler, '90), but the length of synaptic contact in neocortex transplants is very similar to that found in spinal cord transplants (Itoh and Tessler, '90). The percentage of the perimeter of CGRP-labeled synaptic terminals covered by astroglia is similar in transplants of neocortex and cerebellum, but smaller than we have observed in spinal cord transplants (Itoh and Tessler, '90).

DISCUSSION

The principal findings of this study are that, although cut dorsal roots immunoreactive for CGRP regenerate into transplants of fetal brain and establish synapses there, the distribution of the axons is more homogeneous and diffuse than in transplants of embryonic spinal cord, and synapses are far rarer. Embryonic CNS tissues therefore provide an environment conducive to dorsal root regeneration, but the normal target provides additional more specific cues for growth and synapse formation.

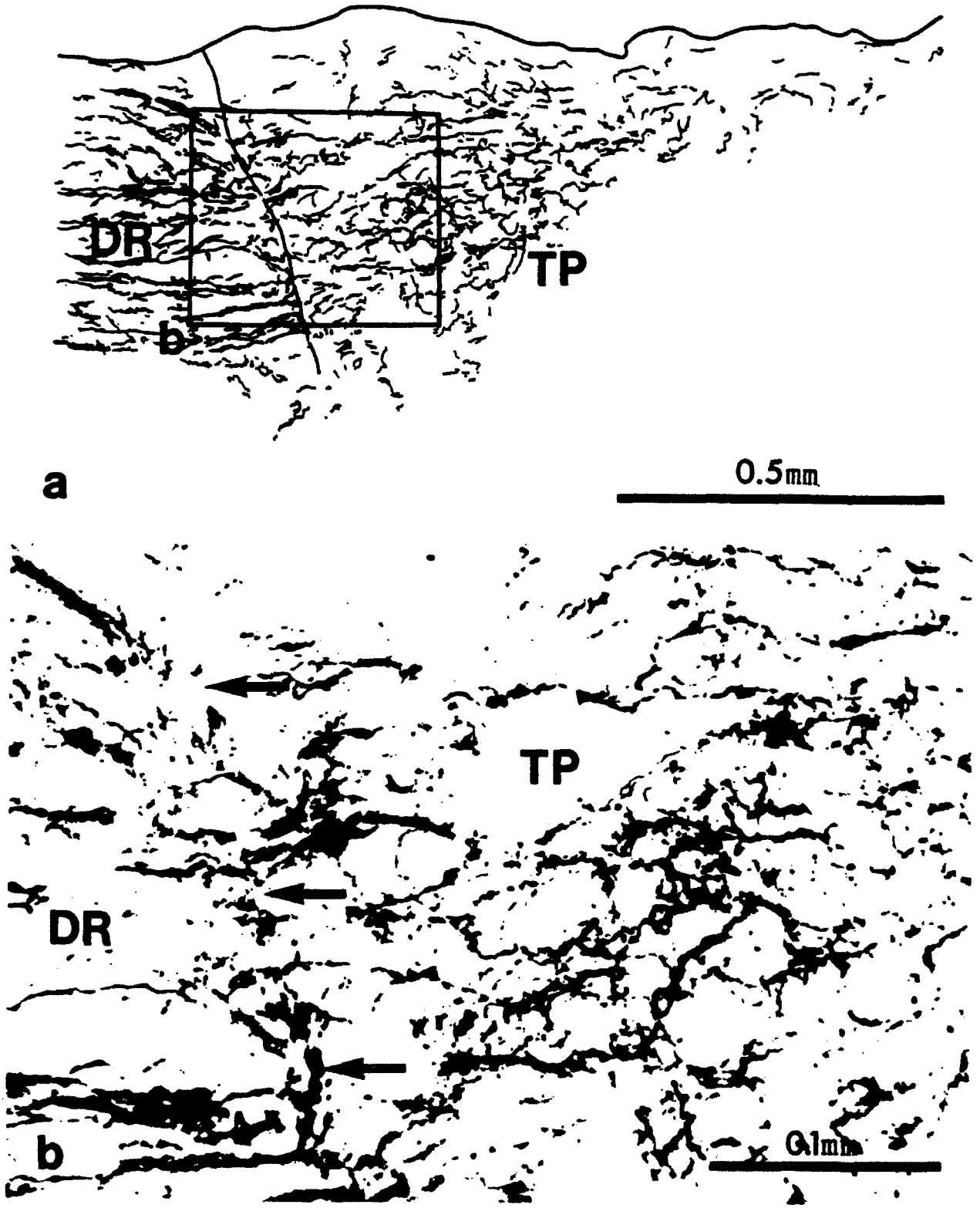

Fig. 11. CGRP-immunoreactive axons in E18 hippocampus graft 1 month after transplantation. Sagittal sections. a: Camera lucida drawing shows primary afferent axons that have crossed the host dorsal root (DR)-transplant (TP) interface and regenerated into the graft. b: A higher-magnification photograph of the interface (arrows) region outlined by the rectangle in Figure 11a shows that axons cross from host dorsal root (DR) into the transplant (TP) and distribute without plexus formation within the graft.

TABLE 2. Comparison of Area Occupied by CGRP-Labeled Axons (Mean ± S.E.M.)

Group		N	Area[1] ($\times 10^4$ μm²)
E14 spinal cord	(A)	5	5.63 ± 0.35
E14 spinal cord-DCS[2]	(B)	5	3.99 ± 0.43
E18 occipital cortex	(C)	5	2.98 ± 0.46
E15 cerebellum	(D)	5	1.01 ± 0.12
Significant differences among groups:[3]			A > B,C > D

[1]Area occupied by CGRP-labeled axons is calculated by point-counting sterological analysis.
[2]DCS: dissociated cell suspensions.
[3]Overall significance determined by one way ANOVA ($p < 0.05$) and individual posthoc comparisons are with Duncan's multiple range test corrected for multiple comparisons ($P < 0.0001$).

TABLE 3. Comparison of Distribution of CGRP-Labeled Axons (Mean ± S.E.M.)

Group		N	Distribution[1] Area (mm²)	Longest axis (mm)	Lateral extension (mm)
E14 spinal cord	(A)	5	0.62 ± 0.06	1.81 ± 0.20	1.14 ± 0.06
E14 spinal cord-DCS[2]	(B)	5	0.98 ± 0.17	1.66 ± 0.22	1.38 ± 0.01
E18 occipital cortex	(C)	5	1.82 ± 0.22	2.34 ± 0.13	1.45 ± 0.07
E15 cerebellum	(D)	5	0.41 ± 0.06	1.27 ± 0.11	0.99 ± 0.10
Significant differences among groups[3]			Area[4]: C > B, A, D[4]		
			Longest axis: C > A, B, D[5]		
			Lateral extension: C, B > A, D[5]		

[1]The distribution of CGRP-immunoreactive axons in the sagittal plane is determined by making montages which consist of all the individual sampling lattices examined.
[2]DCS: dissociated cell suspensions.
[3]Overall significance determined by one way ANOVA ($P < 0.05$) and individual posthoc comparisons are with Duncan's multiple range test corrected for multiple comparisons at the $P < 0.0001$[4], $P < 0.001$[5], and $P < 0.05$[6] levels.

CGRP as a marker for regenerated dorsal root axons

We used CGRP immunoreactivity to demonstrate regenerated dorsal root axons because at least as many DRG neurons are immunoreactive for CGRP as for any other marker described to date (reviewed in Kai-Kai, '89) and because we have found it to be a more sensitive indicator of regenerated axons than methods that rely on axon transport or diffusion of HRP (Tessler et al., '88). Approximately 50% of DRG neurons, primarily those that are small- and medium-sized, are labeled for CGRP, and these are the source of a population of the unmyelinated and thinly myelinated axons that project to the normal dorsal horn (Rosenfeld et al., '83; Gibson et al., '84). CGRP is also found in motoneurons (Gibson et al., '84; Skofitsch and Jacobowitz, '85), but it appears distinctly different. Regenerated axons are readily distinguished in spinal cord transplants because CGRP-immunoreactive perikarya in transplants are rare and devoid of processes (Tessler et al., '88). In the present material we did not find CGRP-immunoreactive perikarya in transplants of cerebral cortex, hippocampus, or cerebellum. Because CGRP-immunoreactive cell bodies and processes are lacking in normal occipital cortex (Rosene and Van Hoesen, '87), all of the labeled axons present in occipital cortex transplants derive from the DRG. CGRP-immunoreactive cell bodies have been reported in Purkinje cells of the normal cerebellum (Kawai et al., '85; Kubota et al., '87; Kruger et al., '88a) and in neurons of the hippocampal formation (Skofitsch and Jacobowitz, '85; Kawai et al., '85). Cerebellar Purkinje cells did not show detectable labeling with a probe for CGRP mRNA (Rethelyi et al., '89), and some of the staining reported in large cerebellar and hippocampal neurons may be artifactual (Kruger et al., '88a,b). Some immunoreactive axons that we observed in transplants of cerebellum and hippocampus may nevertheless derive from neurons intrinsic to the transplants. Their number must be small, however, since we could generally follow the course of the regenerated axons as they passed from the dorsal root into the transplant.

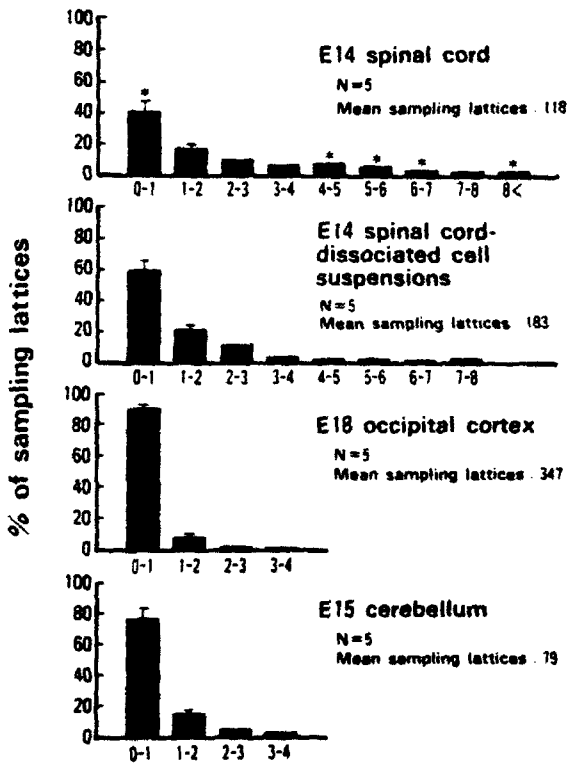

Fig. 12. Distribution histograms showing the area occupied by CGRP-labeled axons per individual sampling lattice (2.6×10^4 μm²) in the four types of transplants. In brain transplants (cortex and cerebellum) the distribution of regenerated CGRP axons is homogeneous but sparse. In contrast, in spinal cord, the ingrowth of CGRP axons is more dense, but there is greater heterogeneity in their distribution, which varies from < 1 to > 8 × 10^3 μm² per sampling lattice.

Regeneration into embryonic brain transplants

CGRP-immunoreactive dorsal roots regenerated into all the embryonic brain regions that we provided as targets: transplants of E14 and E18 neocortex, E18 hippocampus,

TABLE 4. Comparison of Dorsal Root Stumps (Mean ± S.E.M.)

Group	N	Contact length[1] (mm)	Diameter[2] (mm)	Width[3] (mm)
E14 spinal cord	5	0.47 ± 0.06	0.26 ± 0.03	1.01 ± 0.07
E14 spinal cord-DCS[4]	5	0.48 ± 0.06	0.29 ± 0.03	1.24 ± 0.03
E18 occipital cortex	5	0.58 ± 0.10	0.24 ± 0.02	1.15 ± 0.07
E15 cerebellum	5	0.69 ± 0.11	0.33 ± 0.05	1.03 ± 0.12

[1]The length of contact at dorsal root-transplant interface.
[2]The diameter of the dorsal root 750 μm from the interface.
[3]The width of the dorsal root apposed to the transplants.
[4]DCS: dissociated cell suspensions.
No significant differences among groups ($P > 0.05$).

Fig 13. Electron micrographs from E14 neocortex transplants 1 month after transplantation. Bars = 1 μm. **a:** An example of an asymmetric axodendritic synapse (arrow) in which a presynaptic profile containing spherical and dense cored vesicles contacts a dendrite (D). **b:** A CGRP-immunoreactive complex presynaptic terminal containing dense-cored and spherical vesicles makes asymmetric synaptic contacts (arrows) upon different dendritic profiles (D).

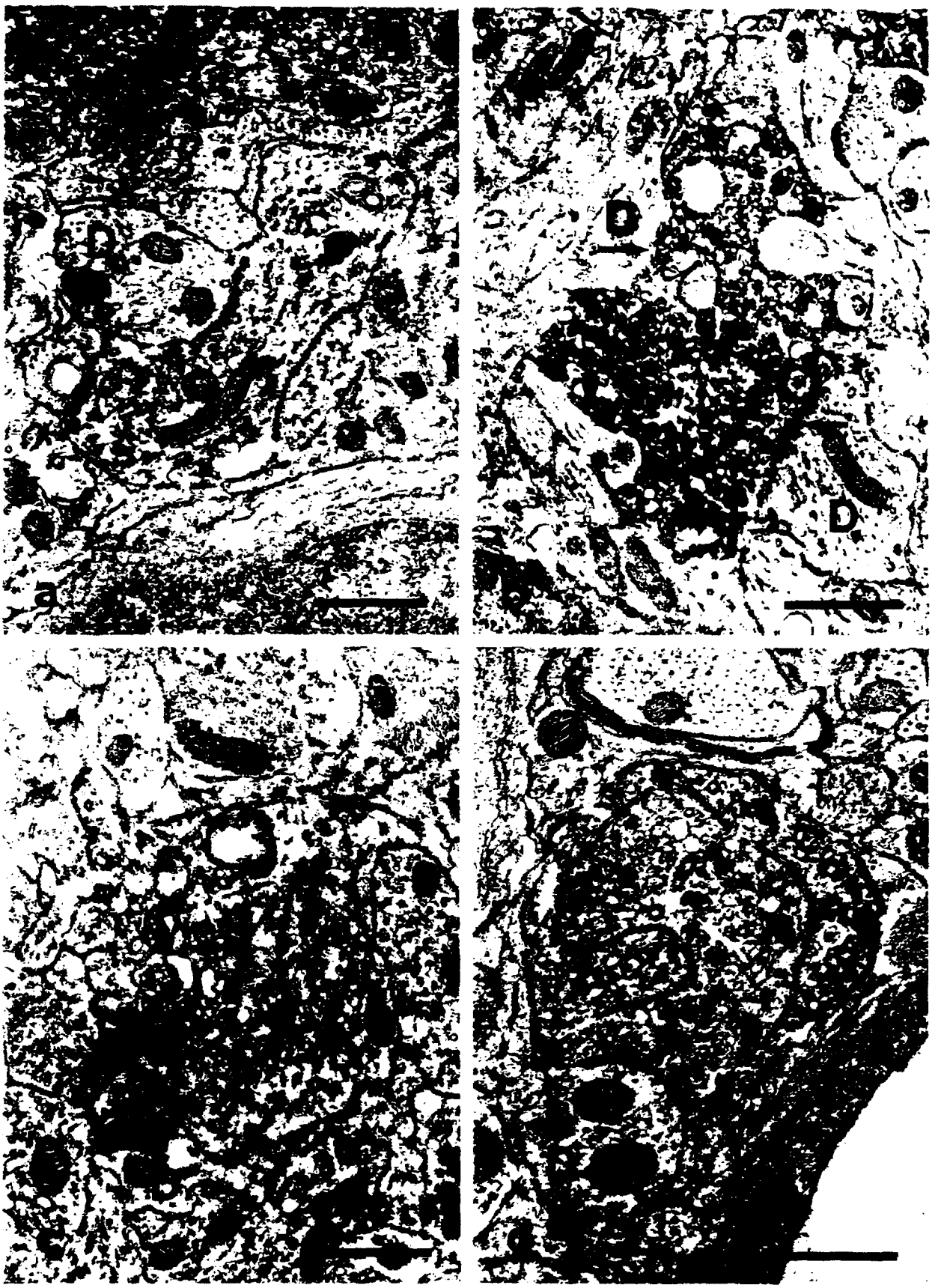

Fig. 14. Electron micrographs from granule cell (a and b) and molecular (c and d) layers of E15 cerebellum grafts 1 month after transplantation. Bars = 1 μm. **a:** An example of a terminal that contains spherical and dense cored vesicles, and makes an asymmetric contact (arrows) upon a dendritic profile (D). **b:** An example of a complex terminal that contains strongly CGRP-immunoreactive spherical and dense cored vesicles, and makes two asymmetric contacts (arrows) upon different dendritic profiles (D). **c:** A presynaptic terminal containing spherical vesicles makes two asymmetric contacts (arrows) upon different dendritic profiles (D). **d:** A CGRP-immunoreactive presynaptic terminal makes an asymmetric synaptic contact (arrow) upon a dendritic profile (D)

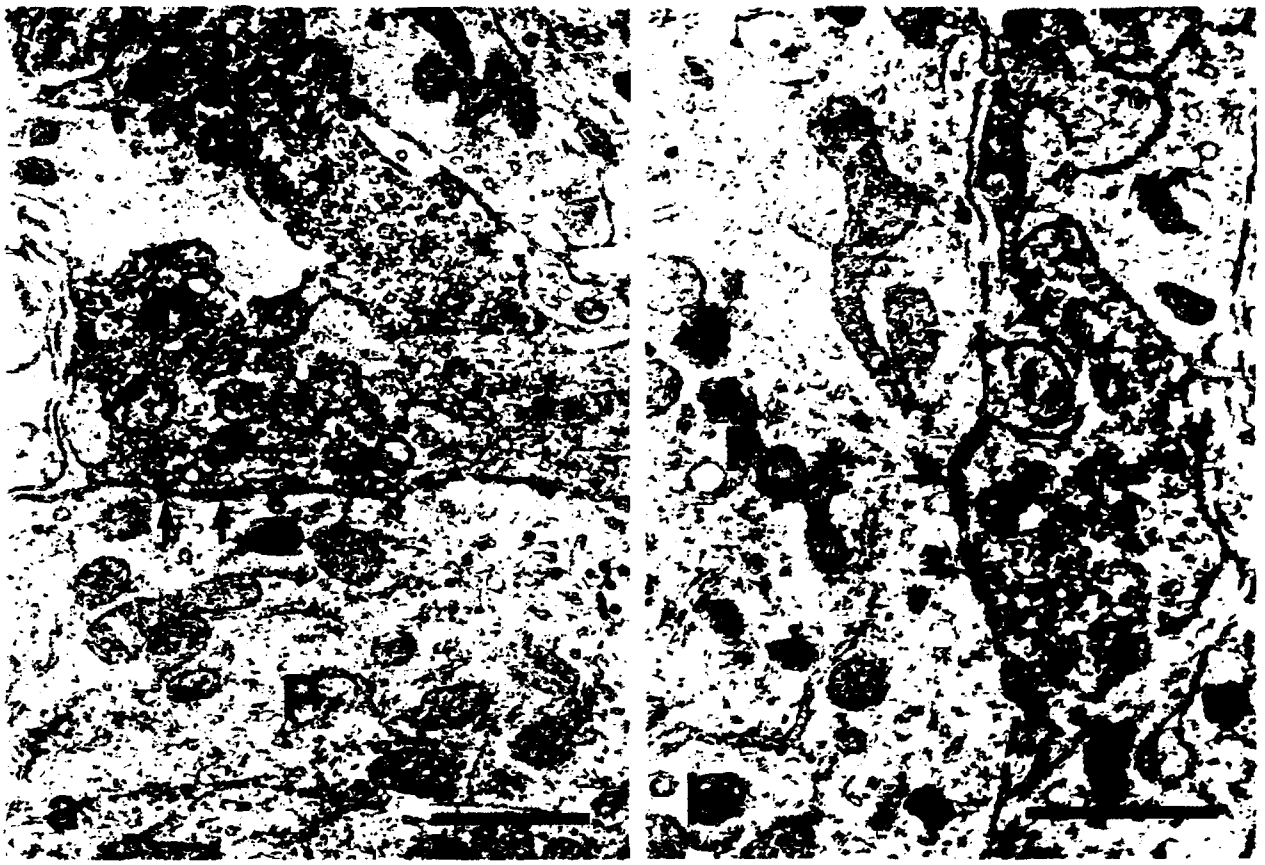

Fig. 15. Electron micrographs from E14 neocortex (a) and E15 cerebellum (b) grafts 1 month after transplantation. Bars = 1 μm. **a:** A CGRP-labeled axon terminal contacts the perikaryon (P) of a cortical neuron by puncta adherentia (arrows). **b:** An axon terminal containing CGRP-labeled spherical and dense-cored vesicles contacts the perikaryon (P) of a granule cell by typical puncta adherentia (arrows).

and E15 cerebellum. Growth patterns, however, were not the same. Like peripheral nerve grafts (reviewed in Aguayo, '85), CNS tissues that are not normal targets can still support or enhance the growth of severed axons. Growth may in part be due to the lack of inhibition (Liuzzi and Lasek, '87; Caroni and Schwab, '88). Transplants from all of these regions are also likely, in addition, to provide conditions that encourage axon elongation. Cell surface and substrate molecules as well as diffusible chemotropic factors are among the molecules that are known to support axon growth (reviewed in Purves and Lichtman, '85). It is unlikely that inappropriate targets taken from diverse areas of brain reproduce precisely the conditions found in the embryonic spinal cord. Growth into inappropriate targets is therefore consistent with the concept that the early stages of axon extension depend on molecules that are expressed generally throughout the developing nervous system (reviewed in Jessell, '88). It is also consistent with in vitro evidence that suggests redundancy in the cues that support axon growth (Bixby et al., '88). CNS neurons differ, however, in the extent to which they regenerate into peripheral nerve grafts (Aguayo, '85), and transplants differ in the amount of regeneration that they elicit from a single population of neurons (Harvey et al., '87; Yoon, '79). Both the growth requirements of severed axons and the factors provided by the substituted targets may differ, and the way in which cut axons grow in response to a particular transplant will depend on the degree to which the transplant satisfies the requirements of these axons for growth. The transplants that we studied also differed in their capacity to support or stimulate growth. Our qualitative observations indicated that growth into transplants of occipital cortex, where there are normally no CGRP projections, exceeded growth into hippocampus, and virtually all of our quantitative results showed more extensive growth into neocortex than cerebellum transplants. The transplant properties that sustain growth of regenerating dorsal roots are unknown, however, and therefore whether or not the same mechanisms or combination of mechanisms account for growth in the various brain regions is also unknown.

TABLE 5. Comparison of Synaptic Terminals (Mean ± S.E.M.)

Group	N	Area (μm)	Perimeter (μm)	Longest axis (μm)	Length of SC[1] (μm)	Glia per perimeter (%)
E15 neocortex	2[2]	4.32 ± 0.52	12.24 ± 1.08	4.20 ± 0.38	0.77 ± 0.10	18.84 ± 2.95
E15 cerebellum	3[3]	2.82 ± 0.31	10.58 ± 0.91	3.42 ± 0.24	0.66 ± 0.05	15.92 ± 2.66

[1]Synaptic contacts.
[2]Data from 24 CGRP-labeled synaptic terminals.
[3]Data from 45 CGRP-labeled synaptic terminals.

Synapse formation in embryonic brain transplants

Regenerated CGRP-immunoreactive dorsal root axons established synapses in the two occipital cortex and three cerebellum transplants that we examined ultrastructurally. Like cut retinal ganglion cell axons (Zwimpfer et al., '89), regenerated DRG axons therefore have the capacity to form synapses with neurons in inappropriate targets. Although factors in the target are thought to determine most of the features of synapse morphology (Campbell and Frost, '88), none of the relatively few that we encountered had the unique appearance of mossy fiber rosettes or basket cell terminals in normal cerebellum. Most, in both neocortex and cerebellum transplants, formed asymmetric (Gray's type I, '59) contacts with a single dendrite, and some formed complex synapses with two or more postsynaptic profiles.

Comparison with regeneration and synapse formation in spinal cord transplants

Except that they were larger in neocortex transplants (see also Zimpfer et al., '89), synapses in the inappropriate targets resembled those found in normal dorsal horn and in transplants of E14 spinal cord (Itoh and Tessler, '90). Regenerated CGRP-labeled axons formed fewer synapses in brain transplants than in transplants of embryonic spinal cord. We rarely found more than 1–2 labeled profiles per grid square in CGRP-innervated regions of brain transplants, whereas the density of labeled synapses in CGRP-rich areas of spinal cord transplants (2.22 per 100 μm^2) is similar to that of normal lamina I (Itoh and Tessler, 1990). If a small percentage of cerebellar neurons synthesize CGRP (Kawai et al., '85; Kruger et al., '88a), then some labeled terminals that we observed in cerebellum transplants may derive from intrinsic cerebellar neurons and those originating from dorsal root axons may be even fewer than our results suggest.

The pattern and extent of ingrowth differed between transplants of spinal cord and brain. In spinal cord transplants regenerated axons stayed relatively close to the host dorsal root/transplant interface, arborized extensively, and were often tangled together in plexuses. Areas of dense ingrowth were separated by regions in which axons were few and scattered. In transplants of brain regions, regenerated axons were distributed widely and sparsely, and grew as individual axons rather than in bundles or plexuses. These qualitative morphological observations were confirmed by the results of our quantitative studies. The area occupied in transplants by CGRP-immunoreactive axons and the density of the regenerated axons were significantly greater in solid pieces of spinal cord than in neocortex or cerebellum transplants. Distribution histograms of the area occupied in transplants confirmed that regenerated axons were distributed sparsely but homogeneously in transplants of brain, whereas spinal cord transplants were heterogeneous for CGRP-labeled axons and contained areas in which growth was dense or sparse. In contrast, several measurements of the extent of distribution within transplants, including area, longest axis, and length of lateral extension, indicated that CGRP-labeled axons spread more widely in cerebral cortex transplants than in solid transplants of spinal cord or cerebellum.

Several explanations for these differences in growth can be excluded. It is unlikely, for example, that the differences are due to the apposition of different numbers of axons to the brain and spinal cord transplants. We observed no difference between brain and spinal cord transplants in the width or length of contact between cut dorsal root and transplant and no difference in the diameter of the juxtaposed dorsal roots measured 750 μm from the transplants. Differences in transplant size also cannot be responsible. Although transplants of cerebellum grew to a smaller size than transplants of cerebral cortex or of spinal cord, our measurements of axon distribution were independent of transplant size. We measured all labeled axons in the transplants, and none of these axons, even in the smallest transplants, reached the margins of a transplant. We cannot exclude that some difference in growth was due to differences in the age or stage of development at which the brain (neocortex, E14, E18; cerebellum, E15; hippocampus, E18) and spinal cord (E14) tissues were transplanted. Neurons in these regions are generated, mature, and establish synapses according to unique developmental timetables (reviewed in Jacobson, '78). At least some systems of afferent axons have invaded these tissues at or within 48 hours of the time of transplantation (Lund and Mustari, '77; Loy and Moore, '79; Zimmer and Haut, '78; Smith, '83; Mason, '86; Rajaofetra et al., '89), however, and neurons are present that might serve as targets for the regenerating dorsal roots (Angevine, '65; Nornes and Das, '74; Lund and Mustari, '77; Altman and Bayer, '78). In addition, the 1-month survival time that we studied would encompass the period during which all of the neurons are born and synaptogenesis is completed (reviewed in Jacobson, '78) if the transplanted tissues develop according to the normal timetable of brain and spinal cord. The availability of targets and the prolonged survival might reasonably be expected to compensate for the several-day differences in age at transplantation.

Different patterns of ingrowth and differences in synaptic number imply that spinal cord and brain provide different signals that affect growth and target recognition within the transplants. Pathway selection and synapse formation are likely to require interactions between growing axons and target structures that are more selective than those that operate during the early stages of axon extension (Jessell, '88; Keller et al., '89). Our observations that regenerated DRG axons grew more densely and formed more abundant synapses within solid transplants of spinal cord than of brain suggest the presence of target-specific cues for pathfinding and target recognition that are not provided by transplants of brain. These results therefore are similar to those of in vitro studies showing that neurites of explanted embryonic DRG axons grew and arborized more abundantly within co-cultured explants of spinal cord than of tectum (Smalheiser et al., '81). Because we examined transplants only 1 month after surgery, we do not know whether or not additional dorsal root axons invaded transplants of cerebellum and cerebral cortex, failed to establish synapses, and then retracted (Smalheiser et al., '81).

The differences between the pathfinding and target recognition cues provided by spinal cord transplants and brain transplants are unknown. We found that regenerated dorsal root axons grew less densely in cell suspensions of spinal cord than in solid spinal cord transplants. This is consistent with the idea that growth and target recognition depend on cell surface cues that can be disrupted mechanically. Surface macromolecules likely to mediate the formation of specific pathways include glycoproteins that are expressed

transiently by discrete populations of neurons (Dodd et al., '88). These glycoproteins, such as the limbic system-associated membrane protein (LAMP) (Levitt, '84; Horton and Levitt, '88), are thought to contribute to target recognition rather than axon outgrowth generally (Keller et al., '89). Subsets of DRG neurons are also distinguished by a series of cell surface carbohydrates (Dodd and Jessell, '85; Chou et al., '89). It has been postulated that these molecules contribute to the orderly ingrowth and selective synaptic connections that are apparent from the time that developing dorsal root axons first invade the spinal cord (Smith, '83; Dodd and Jessell, '85). Two developmentally regulated lectins that might serve as receptors for these cell surface carbohydrates are synthesized by DRG neurons and are also found in the dorsal horn (Regan et al., '86). The lectins were undetected or present at very low levels in areas of the CNS that do not receive primary afferent input, including cerebral cortex, hippocampus, and cerebellum (Regan et al., '86). These are the same regions in which we found DRG axon growth to be relatively sparse.

Our results suggest that embryonic tissues from brain as well as spinal cord provide the conditions under which cut dorsal root axons can grow and survive. The conditions that constitute a permissive environment for regenerating axons are therefore relatively nonspecific, but brain regions nevertheless differ in the extent to which they satisfy the requirements for growth of dorsal roots: neocortex exceeds hippocampus and cerebellum. The results suggest that spinal cord transplants supply additional more specific cues for pathfinding and target recognition. When provided with these more specific cues, axons grow and branch profusely, recognize target neurons in the transplants, and establish relatively normal numbers of synapses. We speculate that the regenerated axons have a more limited distribution in transplants of spinal cord than in neocortex because synapse formation causes the axons to cease growth (Bernstein et al., '78; Liuzzi and Lasek, '87), perhaps by down-regulating the synthesis or otherwise modifying neuron receptors for extracellular matrix molecules (reviewed in Tomaselli and Reichardt, '89). In the absence of these more specific cues for growth within targets and synapse formation, axons generally fail to establish synapses and therefore continue to grow. The behavioral consequences of these different types of intraspinal transplants remain to be explored.

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Time Course of Dorsal Root Axon Regeneration Into Transplants of Fetal Spinal Cord: I. A Light Microscopic Study

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ABSTRACT

Cut dorsal root axons regenerate into intraspinal transplants of fetal spinal cord and establish synaptic connections there. The aims of the present study were to describe the progression of dorsal root growth within the transplants and the maturation of transplant morphology and to determine whether the regenerated dorsal root axons persist within the transplants or eventually withdraw.

Embryonic (E) day 14 spinal cord was grafted into the lumbar enlargement of adult Sprague-Dawley rats, and the L4 or L5 dorsal root was cut and juxtaposed to the transplants. The morphology of the transplants was examined from 1 day to over 1 year after surgery, and the regenerated dorsal roots were labeled with immunohistochemical methods to study the subset that contains calcitonin gene-related peptide (CGRP).

Embryonic spinal cord transplants survived and grew within the host spinal cord in over 90% of the animals. Transplant volume increased and the morphology of the transplants matured over the first 12 weeks and then did not change for 48–60 weeks. During the first week the transplants were composed of dissociated neurons, glia, and hematogenous cells with considerable extracellular space between them. Subsequently, the grafted neurons became densely aggregated, and non-neuronal elements such as inflammatory cells and myelin debris disappeared. CGRP-immunoreactive dorsal roots began to regenerate into the transplants within 24 hours, formed dense bundles by 4 days, and were still present at 60 weeks, the longest survival period examined. Myelination of axons within transplants began at 2 weeks. Quantitative analysis showed that the area of the transplants occupied by CGRP-labeled axons and the distribution area of the labeled axons within the transplants increased until 12 weeks and persisted unchanged for over 48 weeks.

These results indicate that regenerated dorsal root axons are permanently maintained within transplants of embryonic spinal cord and suggest that the transplants can contribute to the permanent restoration of damaged intraspinal neural circuits. © 1992 Wiley-Liss, Inc.

Key words: CNS regeneration, embryonic spinal cord transplants, calcitonin gene-related peptide, immunohistochemistry

Cut central axons of dorsal root ganglion (DRG) neurons fail to regenerate into the mammalian spinal cord (Kimmel and Moyer, '47; Moyer and Kimmel, '48; Reier et al., '83, '86, '89; Liuzzi and Lasek, '87; Reier and Houle, '88) if the injury occurs after the first postnatal week (Caristedt et al., '87). The failure has been attributed to constraints imposed by reactive astrocytes present at the transitional zone between dorsal root and spinal cord (Moyer et al., '53; Pindzola and Silver, '90; Liuzzi, '90). Given an appropriate substrate, such as a transplant of embryonic spinal cord or brain, injured dorsal root axons regrow for distances of several mm and form synapses within the transplants (Tessler et al., '88; Itoh and Tessler, '90a,b). These regenerated axons can be reliably identified immunocytochemically and features of their growth studied quantitatively (Itoh and Tessler, '90a,b). Therefore, the dorsal root-fetal spinal cord system provides a system in which the mechanisms by

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which transplants promote or inhibit regeneration of intraspinal axons can be examined and compared with the mechanisms that act during development or peripheral nerve regeneration.

An important step toward understanding the mechanisms that affect regeneration into transplants is to determine the time course of dorsal root ingrowth. This will establish a framework within which the expression of the molecules that affect regeneration can be studied. The distribution and arborization of dorsal root axons that have regenerated into spinal cord transplants have been described after survivals of 1 to 9 months (Tessler et al., '88; Itoh and Tessler, '90a,b), but the time course of regeneration has not been studied. It is unknown, for example, when regenerating axons first enter the transplants, or whether the ingrowth is complete by 3 weeks after transplantation and therefore resembles the time course of normal dorsal root development (Fitzgerald and Gibson, '84). It is also unknown whether the regenerated axons permanently innervate the transplants and therefore whether transplants provide a strategy for reestablishing damaged circuits within the spinal cord.

In this study, we used immunohistochemical methods to label the subset of regenerated dorsal roots that contains calcitonin gene-related peptide (CGRP). Most of the nearly 50% of DRG neurons that have been reported to contain CGRP are small dark neurons with slowly conducting unmyelinated (C) or thinly myelinated (A delta) axons (McCarthy and Lawson, '90). Larger CGRP-containing DRG neurons with more rapidly conducting A alpha/beta axons have also been observed (McCarthy and Lawson, '90). Dorsal rhizotomy removes virtually all CGRP-containing processes from the dorsal horn (Gibson et al., '84; Chung et al., '88), identifying DRG neurons as their source. CGRP has therefore served as a marker for primary afferents in both the normal dorsal horn (reviewed in Willis and Coggeshall, '91) and in transplants of embryonic spinal cord (Tessler et al., '88; Houle and Reier, '89; Itoh and Tessler, '90a,b). We found that these axons regenerate into embryonic spinal cord transplants by 1 day after axotomy, that they continue to grow within transplants for 3 months, and that their distribution then remains unchanged for more than 1 year.

MATERIALS AND METHODS

Forty-seven adult female Sprague-Dawley rats (200–350 g) received transplants, which were studied after postoperative survivals ranging from 1 day to over 1 year. Table 1 summarizes the analyses of transplanted tissue.

Surgical procedures

Host rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride (76 mg/kg), xylazine (7.6 mg/kg), and acepromazine maleate (0.6 mg/kg), and the lumbar enlargement was exposed by a laminectomy of the T13 or L1 vertebra. After transection of the left L4 or L5 dorsal root approximately 2 mm from the dorsal root entry zone, the distal portion of the root was reflected caudally. A hemisection cavity approximately 3 mm in length was aspirated from the left side of the lumbar enlargement. Solid segments of spinal cord were then dissected from embryonic day 14 (E14) Sprague-Dawley rat pups and introduced into the cavity (Reier et al., '86), and the severed dorsal root stump was juxtaposed to the caudal one-third of the dorsal surface of the transplant. In the rats that were to be studied after survivals of 1 week or less, the cut dorsal root was secured between a sandwich of two transplants. In rats with longer survivals, the dorsal root was not further anchored and its cut end simply rested against the transplant. The dural opening was tightly sutured with interrupted 10-0 sutures, the resected vertebral arch and bone chips replaced, and the superficial wound closed in layers. The procedures have been described in detail (Reier et al., '86; Tessler et al., '88; Itoh and Tessler, '90a,b).

TABLE 1 Summary of 46 Transplanted Animals

Postgraft interval	N	Quantitative analysis	CGRP labeling	% of increase graft volume[1] (Mean ± S.E.M.)
1 day	4	—	- - -	—
2 days	4	—	- - -	—
4 days	4	—	- - -	—
1 wk	4	—	-	—
2 wks	5	5	-	114.06 ± 7.95
4 wks	5	5	+ +	182.25 ± 13.33
12 wks	5	5	+ + +	262.08 ± 11.75
24 wks	5	5	+ + +	270.66 ± 12.47
36 wks	5	5	+ + +	249.51 ± 11.99
48–60 wks	5	5	+ + +	236.52 ± 7.69

Significant differences[1] 2 (wks) < 4 < 48, 36, 12, 24

[1]Groups are ranked in ascending order according to means. Those groups underlined by the same line are not significantly different from one another. Those groups indicated by < are significantly different from one another. Overall significance determined by The Kruskal-Wallis one-way ANOVA ($P < 0.05$) and individual posthoc multiple comparisons ($P < 0.05$).

CGRP immunohistochemistry

From 1 day to 15 months after transplantation, the hosts were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 50 ml of normal saline followed by 500 ml of 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Spinal segments containing transplants were removed, dehydrated, and embedded in paraffin. The specimens studied within 1 week postoperatively were also immersed in fresh fixative overnight before dehydration and embedding. To identify regenerated axons, every fifth section (5 μm in thickness) was processed for calcitonin gene-related peptide (CGRP) immunohistochemistry according to the ABC procedure that has been described previously (Tessler et al., '88; Itoh and Tessler, '90b). Sections were incubated in primary antiserum against CGRP (Peninsula Laboratories, Belmont, CA) at 1:8,000 dilution, immersed in biotinylated goat antirabbit IgG and avidin-biotinylated horseradish peroxidase (HRP) complex (Vectastain ABC kit, Vector Laboratories, Burlingame, CA), and then processed for HRP visualization using 3,3'-diaminobenzidine as chromagen.

To evaluate the histology of the transplant and its interfaces with the host dorsal root and spinal cord, adjacent sections were stained with chromoxane cyanine R or luxol fast blue for myelin and counterstained with cresyl violet.

Quantitative light microscopic analysis

The extent to which CGRP-labeled axons regenerated into transplants was measured after survival periods from 2 weeks to over 1 year (Table 1). Five transplants were examined at each survival time. We used a point-counting stereological analysis to measure the area occupied by immunoreactive axons. Sagittal sections that contained CGRP-immunoreactive axons were examined under a light

microscope at a final magnification ×1,000. A micrometer 10 mm × 10 mm in size ($10^4 \mu m^2$) composed of 1-mm grid squares (OC-M H10/10, Olympus, Tokyo) that was fitted in an ocular lens was used as a sampling lattice, and the number of times that CGRP-labeled axons intersected the corners of the grid squares was counted. Four or 5 sections, separated by five-section intervals, which contained the most abundant CGRP immunoreactivity in the transplant, were examined for each transplant and the results were averaged.

To evaluate the arborization of CGRP-immunoreactive axons, we measured the distribution of labeled axons in 4 or 5 sagittal sections by making composite montages that consisted of all the individual sampling lattices examined and the results were averaged. The area density, expressed as the area occupied by CGRP-immunoreactive axons per 1,000 μm^2, was also calculated as [the area occupied by CGRP-labeled axons]/[the distribution area of labeled axons] × 1,000 μm^2. To estimate the final volume of the transplants, the area of every tenth section cut in the sagittal plane and stained with cyanine R or luxol fast blue and cresyl violet was measured and the sum of the areas was multiplied by the distance between sections (50 μm). The initial volume of the transplants was determined by the procedures described previously (Itoh and Tessler, '90b).

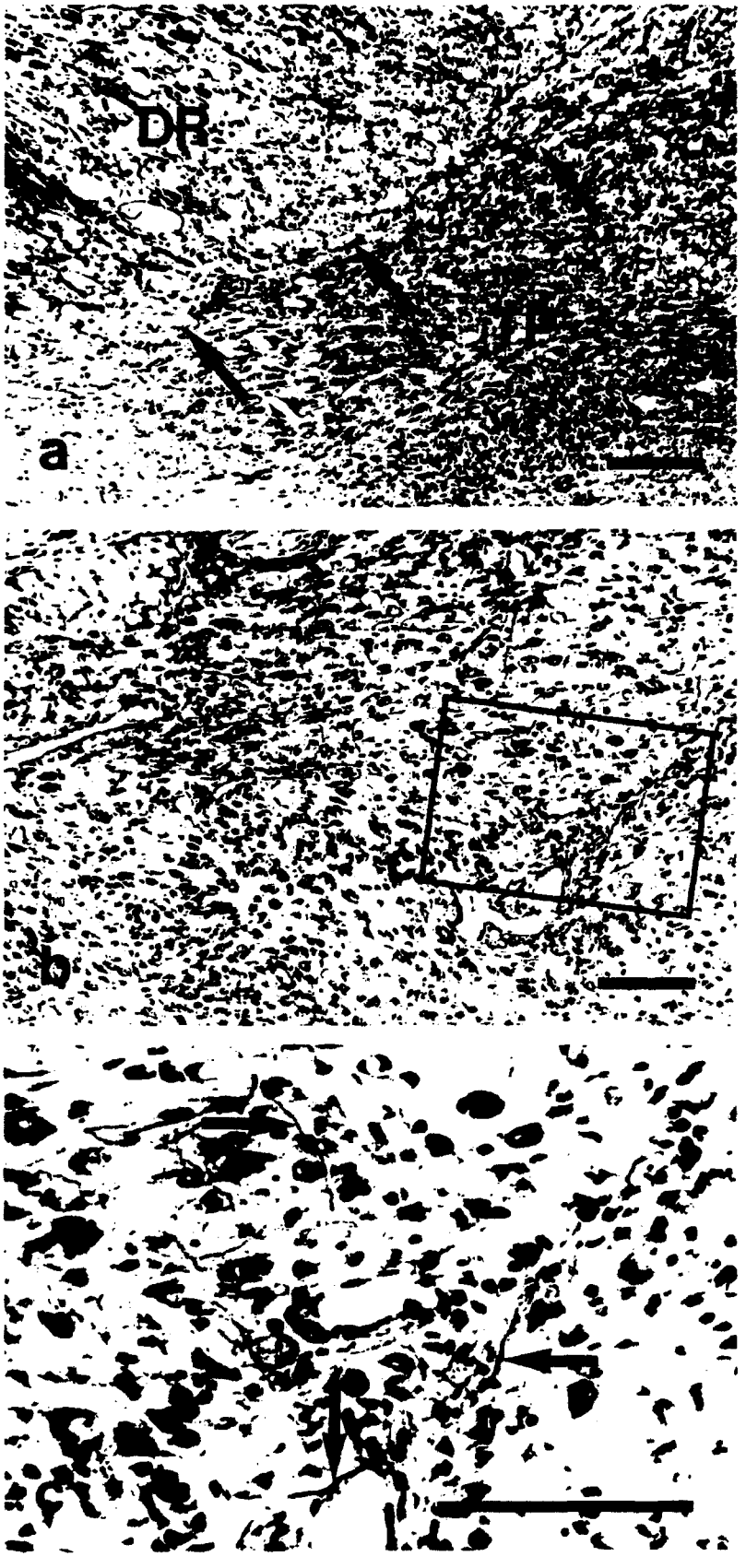
The significance of the differences among transplant groups at various survival periods was evaluated by one-way ANOVA. If there were significant differences ($p < 0.05$) among the groups, each result was corrected for multiple comparisons by using Duncan's multiple range test. The statistical significance of graft volume increases was determined by the Kruskal-Wallis one-way ANOVA corrected with the Wilcoxon-Mann-Whitney test for multiple comparisons. The statistical analyses were performed by using the Number Cruncher Statistical System (Dr. Jerry L. Hintze, Kaysvill, UT).

RESULTS

Transplant development

Over 90% of the hosts had clearly identifiable transplants. In a few instances cavities were present within the transplant, at the interface between host and transplant, or as a dilatation of the central canal of the host spinal cord. At 2 weeks, transplant volume showed a 110% increase over the initial volume, and by 1 month the increase was a statistically significant 180%. The volume continued to increase until 12 weeks and then persisted unchanged for over 48 weeks. The maximum increase of 270% over initial volume was found in 24-week-old transplants (Table 1).

Fig. 1. Sagittal sections of E14 spinal cord graft 24 hours after transplantation. a: A camera lucida drawing shows the relationship of transplant (TP) and host spinal cord (H) and the interface between dorsal root (DR) and transplant. The dorsal root stump is sandwiched between two transplants. Bar = 1 mm. b: A highly magnified photograph of the area outlined by the rectangle in a shows the interface between host dorsal root (DR) and transplant (TP). The neurons are separated by large intercellular spaces and mixed together with non-neuronal elements, including red blood cells, macrophages, lymphocytes, and fragmented myelin (arrows). Stained with chromoxane cyanine R and cresyl violet. Bar = 100 μm. c: Higher magnification photograph of adjacent section shows CGRP-immunoreactive axons (arrows) derived from host dorsal root (DR) regenerated into transplant (TP). Bar = 100 μm.



After 1 or 2 day survivals, spinal cord transplants filled the lesion cavity, but large spaces separated the grafted neurons. Because non-neuronal cells, including glial cells, red blood cells, macrophages and lymphocytes, were present along with the neurons, the transplants were highly cellular and had a loose extracellular matrix (Fig. 1b). In some animals, the lesion cavity was filled with blood, presumably originating from blood vessels cut during the operative procedures along the cut dorsal roots and surrounding tissue of the cavity. The severed dorsal root was demyelinated close to its cut edge, and myelin fragments were observed at the dorsal root-transplant interface (Fig. 1b).

Transplant morphology changed over the 1–2 weeks after surgery. At 4 days, the cells within the transplants were more densely aggregated and had less extracellular space than at 1 and 2 days, but blood cells remained. After 1 week, the blood had largely been resorbed, and macrophages, lymphocytes, and fragments of injured myelin were no longer observed. Spinal cord transplants at this time included dense aggregations of neurons, which showed a high nucleus/cytoplasm ratio and were in some areas arranged in irregular rows. Glial cells and blood vessels were present, but no myelin staining was seen within the transplants (Fig. 2a). By 2 weeks, neurons of different sizes were present throughout the transplants, and myelinated processes were found among the larger neurons (Fig. 2b,c). A dense population of glial cells surrounded the transplants at 2 weeks, especially at the dorsal root-transplant interface, but within the transplants, glial cells were uniformly distributed among the neurons.

After this period, the transplants resembled those that have been described at longer survival times (Bernstein et al., '84; Reier et al., '86; Tessler et al., '88; Jakeman et al., '89; Itoh and Tessler, '90a,b; Jakeman and Reier, '91). They included small and medium-sized neurons similar to those found in the intermediate gray and dorsal horn of the normal adult spinal cord (Reier et al., '86). The extent of myelination within

Fig. 2. Sagittal sections of E14 spinal cord grafts 1 week (a) and 2 weeks (b,c) after transplantation. Nissl-Myelin stain. **a:** The transplant (TP) is well-integrated with host dorsal root (DR) at the interface (arrows). Neurons are densely aggregated, show a high nucleus/cytoplasm ratio, and are partially arranged in irregular rows. The neuropil is immature and does not stain for myelin. **b:** Neurons of different sizes are present throughout the transplant. **c:** A highly magnified photograph of the area indicated by the rectangle in b shows myelinated processes (arrows) among the neurons. Bars = 100 μm.

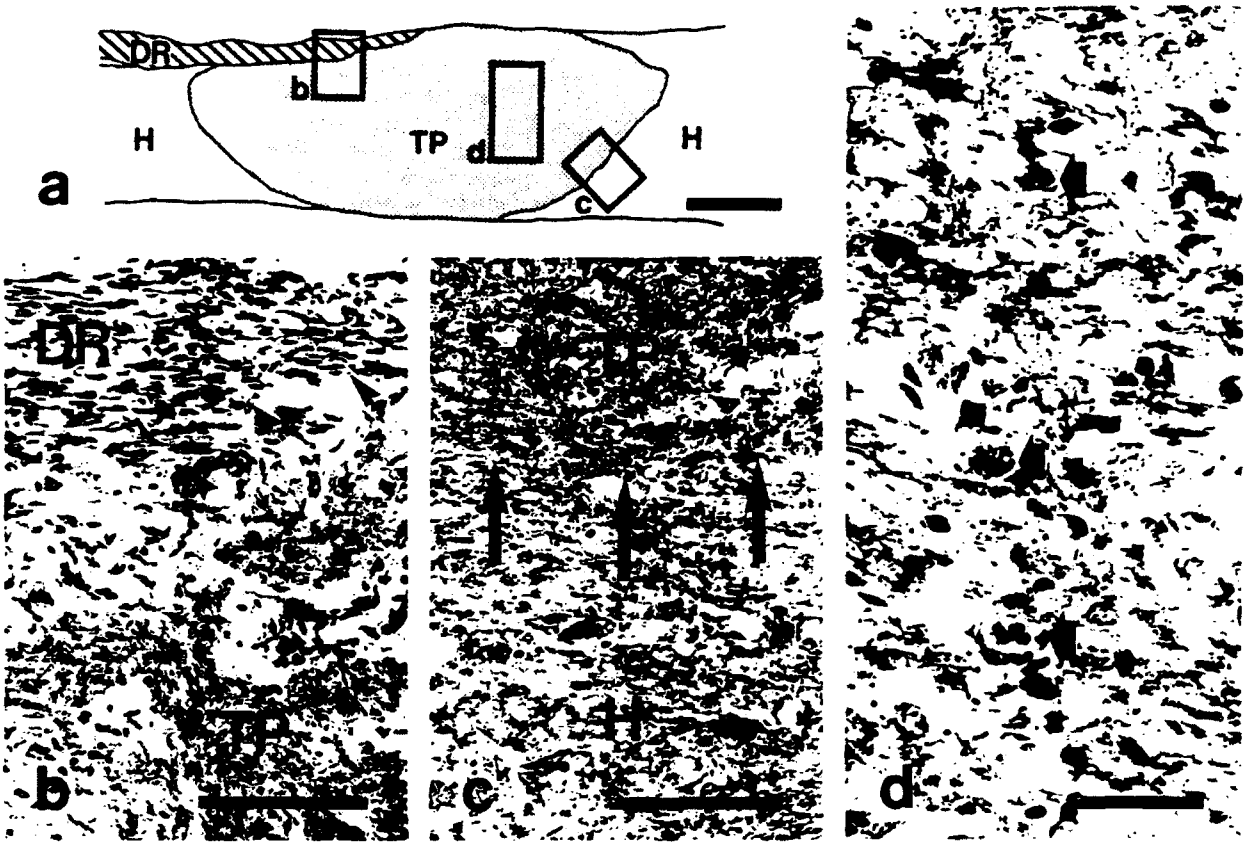


Fig. 3. Sagittal section of E14 spinal cord graft 60 weeks after transplantation. **a:** A camera lucida drawing shows relationship of transplant (TP) and host spinal cord (H) and the interface (arrowheads) between dorsal root (DR) and transplant. Bar = 1 mm. **b:** A highly magnified photograph of the area indicated by the rectangle in a shows the interface (arrowheads) between dorsal root (DR) and transplant (TP). Bar = 10 μm. **c:** Detail of the area outlined by the rectangle (c) in a shows that transplant (TP) and host spinal cord (H) are well-integrated. Bar = 10 μm. **d:** A higher magnification photograph of the region indicated by the rectangle in a shows medium-sized neurons (arrows) surrounded by myelinated axons. Stained with luxol fast blue and cresyl violet. Bar = 10 μm.

the transplants appeared to increase until 12 weeks and then remained unchanged. Myelin was distributed uniformly throughout mature grafts except in distinct myelin-free areas (Reier et al., '86; Jakeman et al., '89). The interface between host white matter and well-myelinated regions of the transplants was difficult to distinguish because in some areas they were not separated by prominent layers of glial cells or their processes (Fig. 3).

CGRP immunohistochemistry

Regenerated dorsal root axons entered the transplants within 24 hours after surgery. The dorsal root and transplant appeared loosely apposed, but a few axons immunoreactive for CGRP had grown into the transplant and extended up to 150 μm from the ends of the cut root stumps (Fig. 1c). By the fourth day more CGRP-immunoreactive axons were present within the transplants and some had grown up to 700 μm from the dorsal root-transplant interface. Varicosities were first observed along the regenerated axons at this time (Fig. 4). Close to the interface with the dorsal root, the CGRP-labeled axons had begun to form the dense bundles that are a characteristic arborization pattern in mature spinal cord transplants (Tessler et al., '88; Itoh and Tessler, '90a,b). The bundles were infrequent at this stage but common in 1-week-old transplants, where they were still located very close to the dorsal root interface. At 4 weeks, the densely aggregated axons had penetrated more deeply into the transplants, and CGRP-immunoreactive axons were less commonly observed immediately adjacent to the dorsal root interface (Fig. 5a). Displacement of these axons from the interface suggests both axon growth and increasing thickness of the layers of astrocytes that are known to develop at the dorsal root-transplant interface (Carlstedt et al., '89; Itoh, '91).

CGRP-containing dorsal roots arborized extensively near the surface of 4-week-old transplants (Tessler et al., '88; Itoh and Tessler, '90a,b) (Fig. 5a), but the extent of the arborization continued to increase until 12 weeks (Fig. 5b). After this period, the extent and the pattern of the regenerated axons persisted unchanged for over 1 year. At 15 months, the longest survival period examined, regenerated CGRP-immunoreactive fibers were clearly identified within the transplants and formed dense plexuses close to the dorsal root-transplant interface (Fig. 6).

Quantitative light microscopic analysis

The stereological analysis showed the area fraction occupied by regenerated dorsal root axons immunostained for CGRP as the transplant developed (Table 2, Fig. 7). At 2 weeks survival, these axons occupied a mean area of 0.64 ×

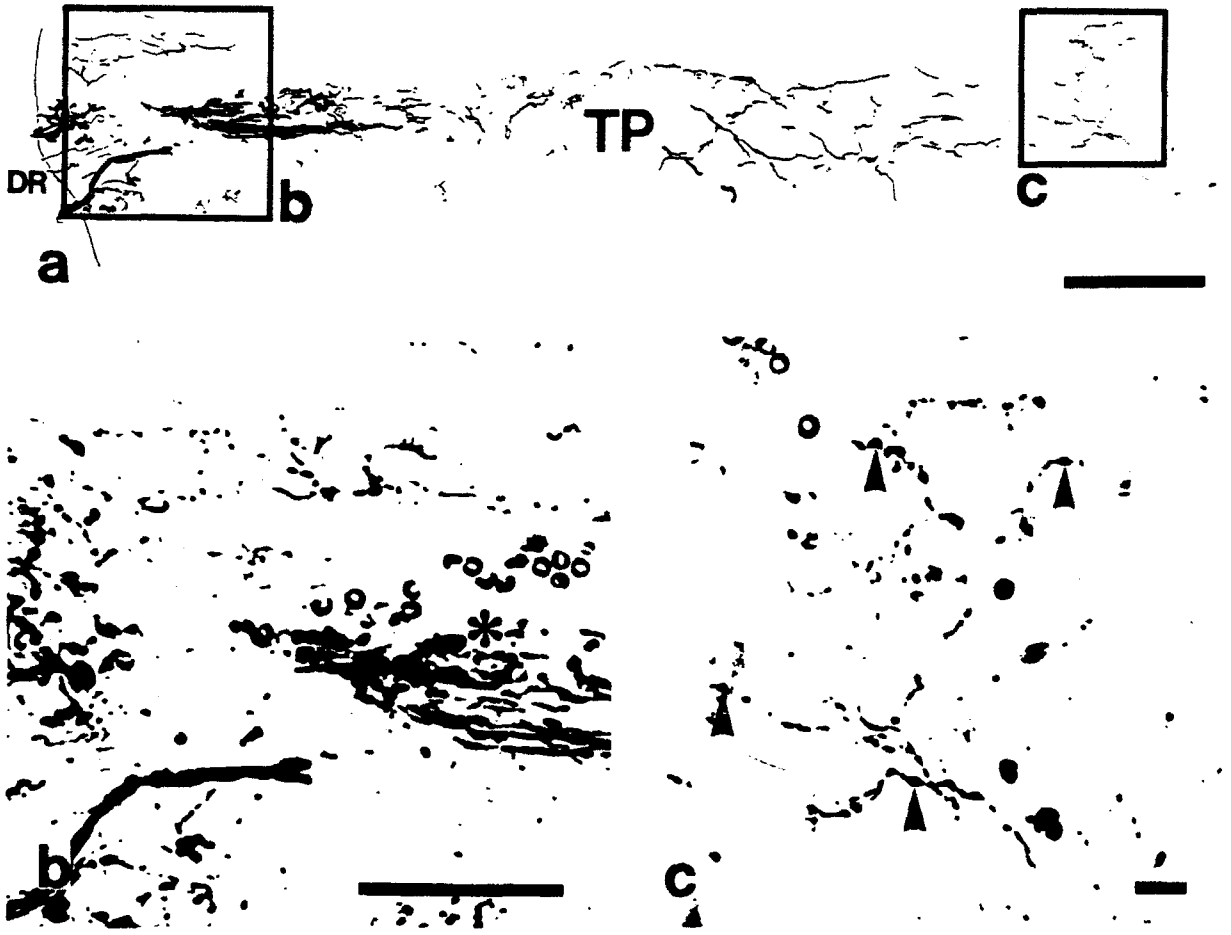


Fig. 4. Sagittal sections of E14 spinal cord graft 4 days after transplantation. a: A camera lucida drawing shows CGRP-labeled axons that have grown across the interface between host dorsal root (DR) and transplant (TP) and regenerated into the transplant. Bar = 100 μm. b: A highly magnified photograph of the area indicated by the rectangle in a shows a dense bundle (*) composed of regenerated CGRP-immunoreactive axons. Bar = 50 μm. c: A more highly magnified photograph of the area indicated by the rectangle in a shows the ends of the regenerated axons. Varicosities (arrowheads) are present along the axons. Bar = 10 μm.

$10^4 \mu m^2$. By 4 weeks, the area fraction occupied was approximately three times greater than at 2 weeks, and at 12 weeks, the increase was approximately sixfold (average area of $4.13 \times 10^4 \mu m^2$). No change occurred thereafter. The area fraction of transplant neuropil occupied by regenerated CGRP-labeled dorsal root axons therefore increased rapidly for the first 3 months and then remained constant for over 1 year.

Table 2 and Figure 7 also show the development of the area within transplants over which regenerated CGRP-labeled axons arborized and the area density of these axons. The mean area of CGRP-innervated regions within 12-week-old transplants was approximately three times that of 2-week-old transplants and twice that of 4-week-old grafts. The regenerated axons therefore extended over a progressively larger area of the transplants for the first 3 months after transplantation and then maintained their area of distribution for more than 1 year. The area density, which represents the area occupied in the transplants by CGRP-labeled axons per 1,000 μm^2 of CGRP-containing area, increased significantly for the first month after transplantation and then persisted unchanged for over 1 year. Together these results suggest that regenerated CGRP-containing dorsal root axons attained their greatest density of distribution in some regions of the transplant by 1 month after transplantation, and that the areas of the transplant occupied to a similar extent continued to increase until 3 months.

DISCUSSION

The major findings of the present study are that severed dorsal root axons of adult rats regenerate into intraspinal embryonic spinal cord transplants within 24 hours after grafting; that the axons continue to extend within the transplants for 3 months; and that the regenerated axons maintain the distribution for over 1 year. Because we have previously shown that regenerated dorsal roots establish synapses within transplants at 1–3 months after surgery (Itoh and Tessler, '90a), the present results suggest that the innervation is permanent.

The three-month period of axon regeneration exceeds the time required for dorsal root ingrowth in the developing spinal cord, which is complete by the second postnatal week (Fitzgerald and Gibson, '84). The explanation for this prolonged period of growth is unclear. Without a trans-

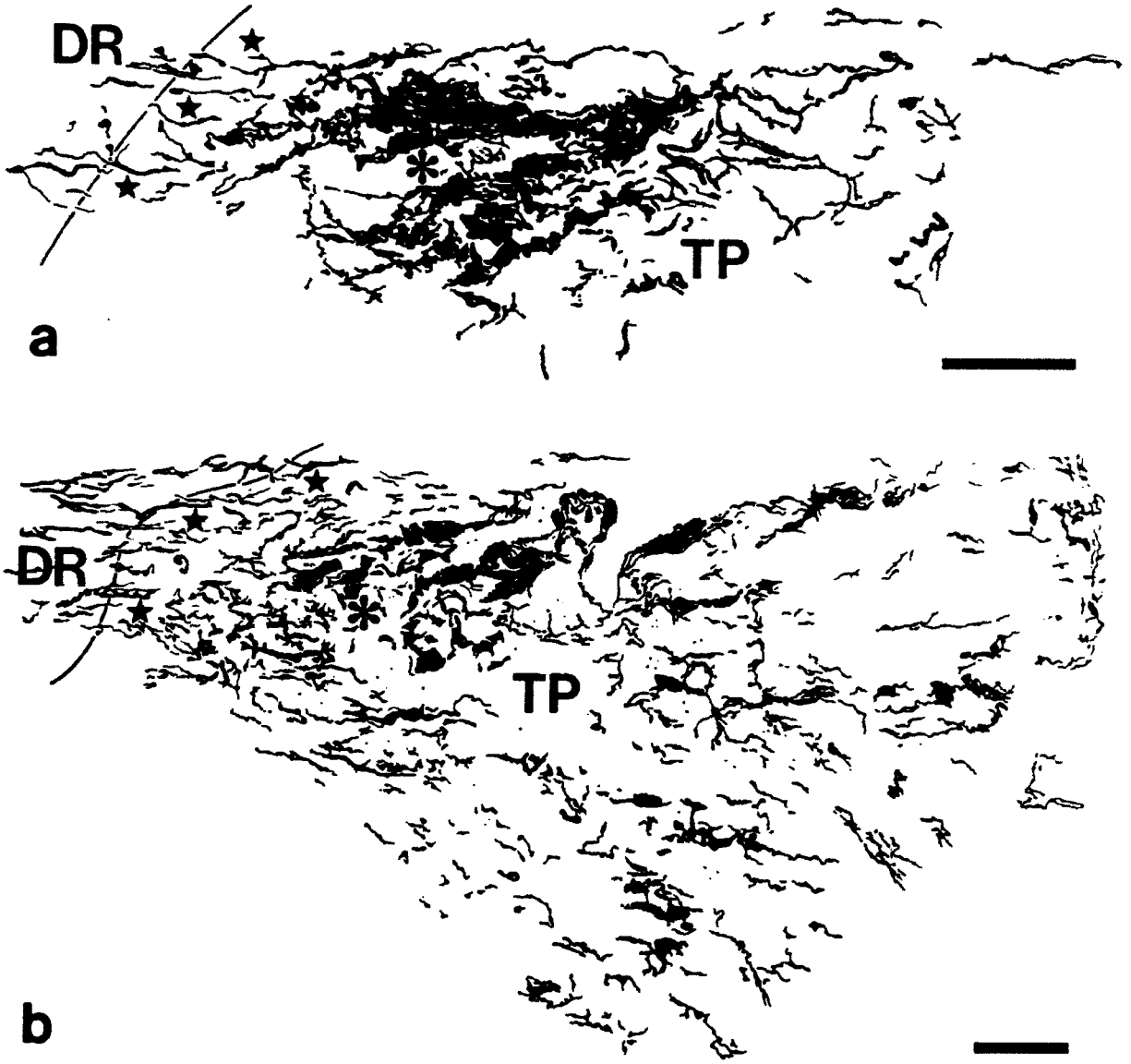


Fig. 5. Sagittal sections of E14 spinal cord grafts 4 weeks (a) and 12 weeks (b) after transplantation. The extent of the arborization of CGRP-labeled axons regenerated into transplants increases between 4 weeks (a) and 12 weeks (b). The distance between host dorsal roots (DR) and the plexuses of regenerated axons (*) in transplants (TP) also increases. Labeled axons are sparse and less branched in the intervening zone (stars) of the transplant. Bars = 100 μm.

plant, axotomized CGRP-containing dorsal root axons are unable to regenerate into spinal cord after the first postnatal week (Carlstedt et al., '87). Astrocytes that develop at the PNS/CNS transition zone are thought to obstruct or inhibit dorsal root regeneration (Moyer et al., '53; Pindzola and Silver, '90; Liuzzi, '90). Our present observations suggest that this barrier is in place by 1 month after transplantation. By this time there is a distinct increase in the distance between host dorsal roots and dense populations of regenerated CGRP-immunoreactive axons, and labeled axons are sparse in the intervening zone. This zone is composed largely of a dense meshwork of hypertrophied astrocytic processes (Carlstedt et al., '89; Itoh, '91). The presence of thick glial layers and few axons immunoreactive for CGRP near the transplant-dorsal root interface is consistent with the idea that primary afferent fibers grow into the transplants prior to the formation of an astroglial barrier that subsequently inhibits axon growth and arborization. Retraction of some of the earlier ingrowing axons may also contribute to the sparcity of immunoreactive axons in the transition zone. Continued entrance of axons from the dorsal roots is, however, unlikely to account for the prolonged period of dorsal root axon regeneration into transplants.

The present study also suggests that dorsal roots grow slowly within transplants. A few CGRP-immunoreactive axons have already regenerated into spinal cord transplants by 24 hours after surgery. Host axons have previously been shown to regrow into intracerebral transplants of embryonic brain by 48 hours (Lund and Mustari, '77; Loy and

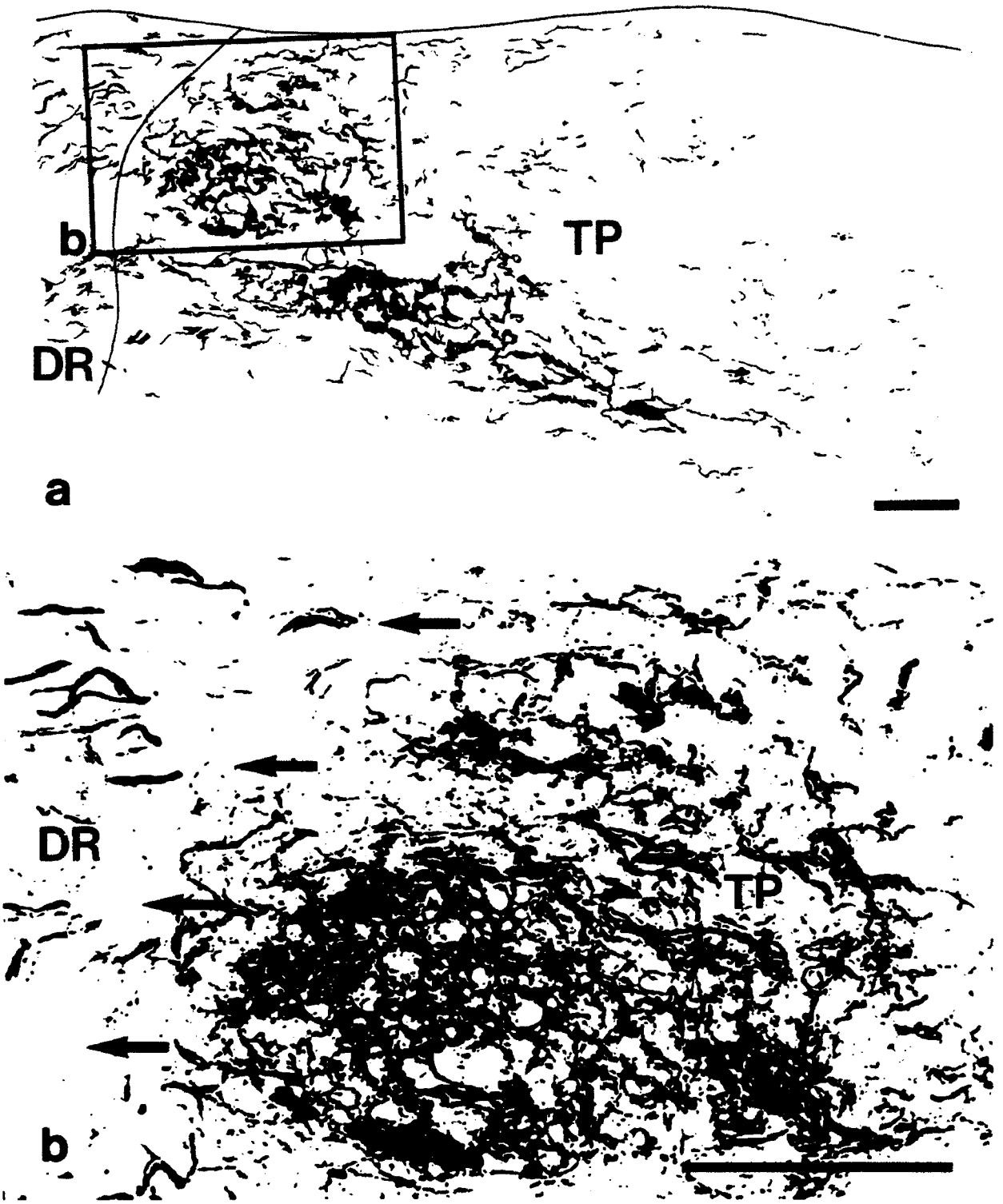


Fig. 6. Sagittal sections of E14 spinal cord grafts 60 weeks after transplantation. a: A camera lucida drawing shows abundant CGRP-labeled axons derived from host dorsal root (DR) that have regenerated into the transplant (TP). b: A more highly magnified photograph of the area outlined by the rectangle in a shows that regenerated axons cross the interface (arrows) between host dorsal root (DR) and transplant (TP) and form a dense plexus within the transplant. Bars = 100 μm.

TABLE 2. Light Microscopic Quantitative Analysis of CGRP-Labeled Axons[1]

Postgraft interval (wks)	Area occupied by CGRP-labeled axons[2] ($\times 10^4/\mu m^2$)	Distribution area of CGRP-labeled axons[3] (mm^2)	Area density[4]
2 (A)	0.64 ± 0.08	0.17 ± 0.01	36.62 ± 3.21
4 (B)	2.18 ± 0.06	0.27 ± 0.02	84.04 ± 6.52
12 (C)	4.13 ± 0.07	0.59 ± 0.02	71.03 ± 2.94
24 (D)	4.25 ± 0.07	0.53 ± 0.03	80.74 ± 4.28
36 (E)	4.40 ± 0.16	0.57 ± 0.03	76.83 ± 1.70
48–60 (F)	4.28 ± 0.11	0.55 ± 0.02	79.10 ± 3.98
Significance[5]	A < B < C,D,F,E	A < B < D,F,E,C	A < C,E,F,D,B

[1]Values are mean ± S.E.M.
[2]Area occupied by CGRP-labeled axons is calculated by point-counting stereological analysis.
[3]Distribution area of CGRP-immunoreactive axons in the sagittal plane is determined by making montages which consist of all the individual sampling lattices examined.
[4]Area density is expressed as the area occupied by CGRP-labeled axons per 1,000 μm^2.
[5]Overall significance determined by one way ANOVA ($P < 0.05$) and individual posthoc comparisons are with the Duncan's multiple range test ($P < 0.05$).

Moore, '79; Zimmer and Haut, '78; Smith, '83; Mason, '86; Rajaofetra et al., '89). At both 1 and 4 days, the axons that have regenerated into spinal cord transplants are straight and unbranched. By comparing the length of regenerated CGRP-containing axons after 1- and 4-day survivals, we estimate a growth rate within transplants at this early stage of regrowth to be 6–7 mm/hour. This rate is much slower than that reported for the most rapidly regenerating sensory axons (150–190 mm/hour, Gutmann et al., '42) but similar to that of developing callosal and corticospinal axons as they extend through the cortex (5–6 mm/hour, Kalil and Norris, '92). Faster rates have been reported for developing callosal and corticospinal axons as they grow through corpus callosum and spinal cord (60 mm/hour, Kalil and Norris, '92), for retinal ganglion cell axons regenerating into PNS grafts (40–80 mm/hour, Cho and So, '87; Bray et al., '92), and for several different kinds of embryonic neurons growing in vitro (Davies, '89) and in vivo (Jacobson and Huang, '85; Davies, '87, '89). The arborization of regenerated dorsal roots after 4 days prevented us from calculating the axon growth rate at later times, but it is likely to be even slower. The area occupied in transplants by CGRP-immunoreactive axons and the extension of arborization continue to increase until 3 months, but the total distance covered by the regenerated axons is limited to only a small and superficial portion of the transplants.

The slow rate of extension within spinal cord transplants may indicate a relatively unfavorable environment for axon growth. The molecular environment of the transplants has not been studied: transplants may lack an adequate supply of molecules that support or enhance growth (Snyder and Johnson, '89; Smith et al., '90) or include molecules that inhibit or repulse growing axons (Caroni and Schwab, '88a,b; Schwab and Caroni, '88; Schnell and Schwab, '90; Snow et al., '90a,b). The slow rate of growth compared with the time course of normal dorsal root development might also be due to differences between developing and regenerating axons. For example, integrin receptors for extracellular matrix molecules, which are present on developing axons, are lost with target contact (reviewed in Reichardt and Tomaselli, '91), and their affinity can also be reduced (Cohen et al., '89). Some of these receptors may not be reexpressed after axotomy, or the affinity of those on regenerating axons may be less than those on developing axons. In addition, the slow rate at which axons grow in transplants may be related to the early formation of

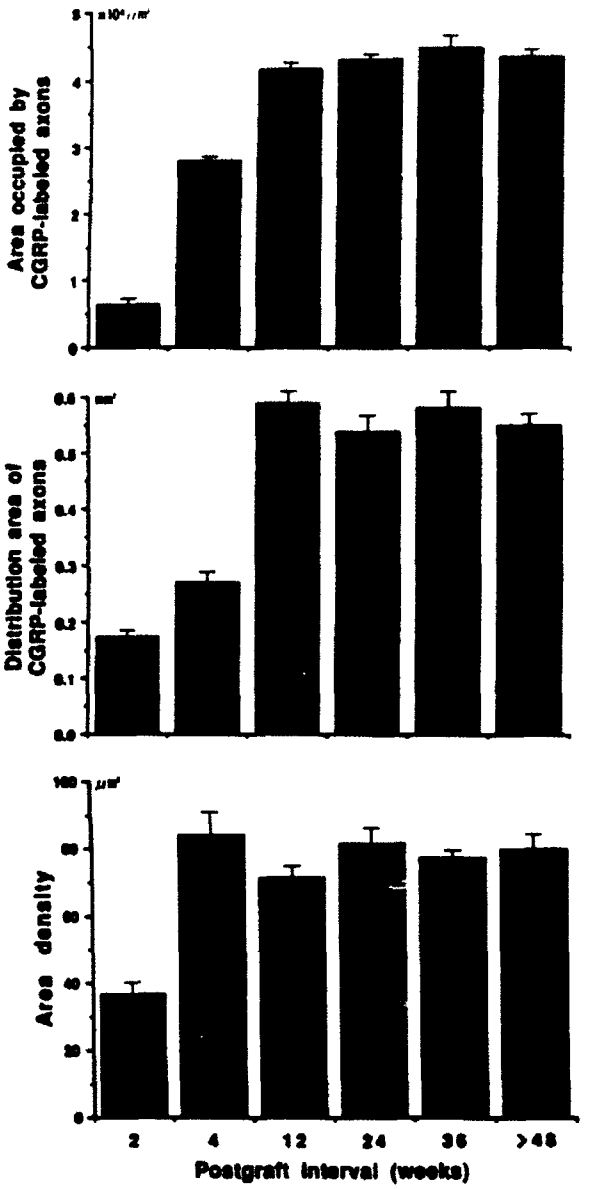


Fig. 7. Quantitative analysis of regenerated CGRP-immunoreactive axons. The area occupied by CGRP-labeled axons and the area of their distribution increase for the first 12 weeks and persist unchanged for over 48 weeks, whereas the area density of CGRP-labeled axons reaches a maximum by 4 weeks. See also text and Table 2. Values are mean ± S.E.M.

synapses (Bernstein and Bernstein, '71), which could decrease the number or affinity of axon receptors for extracellular matrix molecules. We observed varicosities suggestive of synaptic boutons along regenerated dorsal roots by 24 hours, and more of these varicosities were present by 4 days after transplantation.

We cannot exclude the possibility that some of the expansion at longer survival times is due to intraspinal CGRP-containing fibers that derive from Lissauer's tract and lamina X (Houlé and Reier, '89). To insure that regenerated dorsal root axons accounted for the bulk of the growth, however, we limited our analysis to regions close to the apposed dorsal root. Furthermore, because there is

always an area without CGRP-stained axons within the transplants, CGRP-immunoreactive axons that had regenerated into a transplant directly from the cut dorsal root could be distinguished from dorsal root collaterals that derived from the host dorsal horn (Houlé and Reier, '89; Traub et al., '89).

Several mechanisms could account for the prolonged period of dorsal root axon growth within transplants. In part, this growth may be due to branching of the regenerated axons. One condition that may stimulate the formation of axon collaterals is the presence of vacant postsynaptic sites (Raisman, '69; Cotman et al., '81), and we have found that synaptic density in even the most abundantly innervated regions of 1 to 3-month-old transplants is less than that found in lamina I of the normal dorsal horn (Itoh and Tessler, '90a). The prolonged regrowth of DRG axons might also be due at least in part to the absence of signals to terminate growth that derive from normal targets. We have found that regenerated dorsal roots establish a significantly larger number of axoaxonic synapses in spinal cord transplants than is found in normal lamina I (Itoh and Tessler, '90a). Because these synapses are inappropriate, they may not contribute the signals that normally terminate growth. Intrinsic properties of these axons such as their length may also contribute, if neurons with longer axons have the capacity to grow for extended periods of time during regeneration (Fawcett, '92). CGRP-containing DRG neurons include some whose axons ascend to the dorsal column nuclei (McNeill et al., '88; Fabri and Conti, '90).

Our results indicate that regenerated dorsal roots are permanently maintained within fetal spinal cord transplants and, therefore, that these transplants can contribute to the permanent restoration of damaged neuronal circuitry. Other systems of injured axons have been reported to establish longlasting projections into embryonic transplants of their normal target tissues (Bregman and Reier, '86; Bregman, '88). We have found regenerated dorsal roots of adult rats in embryonic transplants of brain as well as of spinal cord after 1 month, suggesting that the conditions that support dorsal root regeneration are not specific to their normal target tissue (Itoh and Tessler, '90b). Target-specific signals may contribute to synapse formation and therefore to the maintenance of projections (Jessell, '88), however, because regenerated dorsal roots make many fewer synapses in transplants of brain than in transplants of spinal cord (Itoh and Tessler, '90b). Whether the permanent establishment of dorsal root projections is target-specific and whether it depends on synapse formation remain to be determined.

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Time Course of Dorsal Root Axon Regeneration into Transplants of Fetal Spinal Cord: An Electron Microscopic Study

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ABSTRACT

Intraspinal transplants of fetal CNS tissue permit or enhance the regeneration of cut central axons of adult dorsal root ganglion (DRG) neurons. Some of these regenerated axons establish synapses with transplant neurons. The aims of the present study were to determine when regenerated DRG axons begin to form synapses with transplanted embryonic spinal cord neurons and whether these synapses are pennanent. We also examined the development of transplant neuropil in areas innervated by the regenerated axons. Whole pieces of embryonic day (E)14 spinal cord were introduced into hemisection cavities made at the level of the lumbar enlargement, and the cut L4 or L5 dorsal root was juxtaposed to the transplant. Regenerated DRG axons immunoreactive for calcitonin gene-related peptide (CGRP) were labeled by immunohistochemical methods and examined by electron microscopy from 1 week to 1 year after surgery. CGRP-immunoreactive axon terminals made synaptic contacts with dendrites and perikarya of transplant neurons by 1 week after axotomy. The morphology of the synapses was immature. Large growth cone-like structures were also present at 1 week but not at 2 weeks or later. At 2 weeks, regenerated unmyelinated axons formed terminals similar to those found in animals surviving for 48 weeks. Axoaxonic synapses in which the pre- and post-synaptic elements were immunolabeled for CGRP and regenerated CGRP-labeled myelinated axons were observed at 4 weeks and later. The area of distribution of CGRP staining increased until 12 weeks and the synaptic density of regenerated CGRP-labeled terminals increased for 24 weeks. The results indicate that the synaptic terminals of regenerated

primary afferent axons are permanently retained within fetal spinal cord transplants. Transplants may therefore contribute to the permanent restoration of interrupted neural circuits.

INTRODUCTION

The cut central processes of adult DRG neurons grow into transplants of embryonic spinal cord (15-20, 40, 44, 46), and form synapses with donor neurons (18-20). Electical stimulation of host dorsal roots evokes extracellular single unit activity (16, 21, 40) and excitatory postsynaptic potentials and action potentials (21, 45) from transplant neurons, indicating functional connectivity. The DRG axon - spinal cord transplant system therefore provides an in vivo model for studying the mechanisms by which the transplant environment supports or enhances regeneration.

The ultrastructural organization of intraspinal fetal spinal cord transplants has been described (2, 19, 23, 35, 39), but the time course of development of the transplanted tissue and the time course of the maturation of the regenerated axons are unknown. It is also unknown when regenerated dorsal root axons establish synapses and whether the innervation is permanent.

DRG neurons are the sole afferent source of the calcitonin gene-related peptide (CGRP) immunoreactivity that is found in the normal dorsal horn (9, 50). Because CGRP-immunoreactivity also provides a reliable marker for regenerated dorsal root axons in transplants of fetal spinal cord (15, 18-20, 46), we have used immunohistochemical procedures to label the subset of regenerated dorsal root axons that is immunoreactive for CGRP. The results indicate that regenerated dorsal root axons form synapses within the transplants by 1 week after axotomy, and that the synaptic terminals remain for at least 48 weeks.

MATERIALS AND METHODS

Twenty-eight adult female Sprague-Dawley rats (200-350g) received transplants of embryonic spinal cord and survived for 1, 2, 4, 12, 24, 36, and 48 weeks postoperatively. Four animals were analyzed at each survival time.

The surgical procedures were identical to those described elsewhere (18). In brief, the animals were anesthetized with an intraperitoneal injection of ketamine hydrochloride (76 mg/kg), xylazine (7.6 mg/kg), and acepromazine maleate (0.6 mg/kg), and the lumbar enlargement was exposed by a laminectomy. Segments of spinal cord were dissected from E14 Sprague-Dawley rat pups and introduced as whole pieces into a hemisection cavity approximately 3mm in length aspirated from the left side of the lumbar enlargement. The L4 or L5 dorsal root was then transected close to the dorsal root entry zone and juxtaposed to the dorsal surface of the transplants. The dura and the superficial wound were closed in layers.

CGRP immunohistochemistry

To determine when regenerated dorsal root axons begin to form synapses within the transplants and to assess the development of regenerated dorsal root axons and synaptic terminals, intraspinal transplants were analyzed after survivals of 1 week to 48 weeks by electron microscopic immunohistochemistry. The procedures for demonstrating CGRP immunoreaction product have been described previously (19, 20).

In brief, the host rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with normal saline followed by a mixture of fixatives containing 3% paraformaldehyde, 3% glutaraldehyde, 0.1% picric acid, and 0.02mM CaCl2 in 0.1M cacodylate buffer at pH 7.4. Spinal segments that contained transplants were promptly removed. Vibratome sections (40 μm in thickness) were cut in the sagittal plane and treated with 1% sodium borohydride in phosphate-buffered saline (26). Following overnight incubation in rabbit anti-human CGRP antiserum (Peninsula Laboratories, Belmont, CA) diluted at 1:16,000, the sections were processed for the ABC procedure, osmicated (41), dehydrated, and flat-embedded in Epon-Araldite mixture.

Stereological analysis of transplant neuropil

To evaluate the development of transplant neuropil in regions that were densely innervated by regenerated CGRP-labeled axons, we performed a stereological analysis using the following sampling procedures.

Two flat-embedded Epon-Araldite sections that contained dense bundles of CGRP-labeled axons and were within 1 mm of the dorsal root-transplant interface were selected from each recipient. Ultrathin sections were cut with a LKB Ultratome IV, placed on thin-meshed 300 grids, and examined in a JEOL 1200EX electron microscope (80kV). The sections were not stained with lead citrate or uranyl acetate. Regions were identified in 2-week-old transplants that contained approximately 5 adjacent grid squares with axonal or terminal profiles immunoreactive

for CGRP; in 4-week-old grafts, 10-15 adjacent grid squares; and in transplants after 12 weeks, at least 15 adjacent grid squares. Two photographs (x6,500 magnification) were taken from alternate grid squares and a total of 10-50 electron micrographs were collected from each recipient. Grid squares in which more than one-half of the field was occupied by blood vessels or by bundles of myelinated axons were excluded from the survey (32). The stereological analysis entailed placing a grid containing 100 intersections over each electron micrograph enlarged to a final magnification of 20,000x. The structures under each intersection were identified as myelinated and unmyelinated axons, axon terminals (profiles containing spherical, spherical and dense cored, or pleomorphic vesicles), cell bodies, dendrites, and non-neuronal structures (glia, blood vessels, and unidentified contours) using the criteria described previously (19, 27, 31, 32). The area fraction occupied by axons and terminals was also classified into CGRP-labeled and CGRP-unlabeled elements as determined by the presence or absence of CGRP immunoreaction product. Because the area and volume of the tissue occupied by each component are directly related to the area fraction estimated by the point counting stereological analysis (47), we were able to calculate an approximation of the area occupied by each component in CGRP-innervated regions. The distribution area of CGRP-labeled axons measured by quantitative light microscopic analysis (data from 18) was multiplied by the area fraction occupied by each component. The results from the number of animals in each survival period were averaged.

To determine the synaptic density within transplants, the number of synapses was counted according to the protocol that has been described previously (19, 43). The area examined on the electron micrographs was

11.7 μm x 8.3 μm. Synaptic density was expressed as the number of synapses per 100 μm^2. Synapses were also classified into CGRP-labeled or CGRP-unlabeled elements as determined by CGRP immunoreactivity in pre- or post-synaptic endings.

Morphometric analysis

Synaptic terminals in electron micrographs used for the stereological analysis were subsequently analyzed morphometrically. A profile was considered a presynaptic terminal if it contained 5 or more synaptic vesicles, formed distinct synaptic complexes with postsynaptic neuronal elements, and had synaptic vesicles closely associated with the synaptic junctions (36).

The area and synaptic contact length of 50-75 synaptic terminals from each transplant were measured using the Bioquant System IV (R&M Biometrics, Inc., Nashville, TN), and the results were averaged. Because few synaptic terminals were identified in 1- and 2-week-old transplants, we studied all that we found in each transplant. Each presynaptic terminal was further classified on the basis of the following morphological characteristics (19): i) immunoreactivity for CGRP: positive or negative; ii) synaptic vesicle content: spherical, spherical and dense cored, or pleomorphic vesicles; iii) number of synaptic contacts: simple if it made only one synaptic contact per profile, or complex if it made two or more; iv) postsynaptic structure contacted: dendritic, somatic, or axonic (see 19 for further details concerning classification criteria). For each terminal we also calculated a multisynaptic index (MSI), which

represents the number of synaptic contacts with separate postsynaptic structures.

To examine the development of myelinated axons within transplants, 30-50 myelinated axons from each transplant were analyzed. Myelinated axon diameter, axonal diameter, thickness of myelin sheath, and thickness of internal mesaxon were measured as the minor axes of elliptical cross sections. The myelin index was calculated as axonal diameter/ myelinated axon diameter (3). Myelinated axons were also classified as CGRP-labeled or -unlabeled.

Statistical significance of the stereological analysis was determined by the Kruskal-Wallis one-way ANOVA. When significant differences were present ($p<0.05$), individual posthoc comparisons were corrected with the Wilcoxon-Mann-Whitney test for multiple comparisons. The significance of the comparisons of the morphometric analysis among the transplant groups of each survival period was evaluated by one-way ANOVA. When there were significant differences ($p<0.05$) among the transplant groups, each result was corrected for multiple comparisons by using Duncan's multiple range test. The statistical analyses were performed on the Number Cruncher Statistical System (Dr.J.L.Hintze, Kaysvill, UT).

RESULTS

CGRP-immunoreactive axons and terminals were readily identified within the transplants. In myelinated and unmyelinated axons, immunolabeling for CGRP was consistently associated with microtubules; in axon terminals, CGRP was associated with dense-cored vesicles. The general morphology of CGRP-labeled axons and terminals observed in the present study was very similar to that which has been described previously in superficial dorsal horn of mammalian spinal cord (4, 5, 9, 19, 30, 41). Because we used a pre-embedding method for CGRP immunohistochemistry, which has limited penetration, the number of CGRP-labeled profiles will be underestimated. Nevertheless, the examination of the labeled profiles enabled us to characterize the entry of a population of regenerated dorsal root axons into the transplants.

Very few CGRP-labeled axons and terminals were present at 1 week, the shortest survival period examined by electron microscopy. The total of 14 CGRP-labeled and 12 CGRP-unlabeled presynaptic terminals that we observed in 4 animals was restricted to a region close to the dorsal root-transplant interface. Some of these regenerated CGRP-immunoreactive axons made synaptic contacts with dendrites and perikarya of transplant neurons at this time (Fig. 1a). Most of these synaptic terminals were entirely surrounded by glial processes and appeared immature because they contained relatively few synaptic vesicles and their pre- and post-synaptic membranes were less well developed than those found in more mature spinal cord transplants (19). Most CGRP-immunoreactive synaptic contacts were asymmetric (Gray's

type 1), and most of the labeled presynaptic terminals contacted one or two dendritic profiles. Some CGRP-labeled axon terminals established two or more individual synaptic contacts, as is commonly found in mature spinal cord transplants (19), but axoaxonic synapses in which the pre- and post-synaptic elements were immunoreactive for CGRP were not identified at this stage. We also did not observe myelinated axons at this time.

An unusual large profile resembling a growth cone was seen only within 1-week-old grafts. These structures were filled with numerous dense-cored vesicles labeled for CGRP, spherical vesicles, mitochondria, and microtubules (Fig.1b). Most were entirely surrounded by glial processes, but some were partly apposed to neuronal elements without interposed glia. Because the profiles were not observed within the grafts at 2 weeks or later, they appeared to be characteristic of the very early stage of dorsal root axon regeneration. Although interrupted serial sections of these profiles did not reveal filopodia and lamellipodia, they are likely to represent growth cones of regenerating dorsal root axons.

At 2 weeks, CGRP-immunoreactive axons and terminals were still infrequent. We found only 27 CGRP-labeled and 60 CGRP-unlabeled synaptic terminals in the 4 transplants that we studied at this stage. Nevertheless, most CGRP-labeled terminals had acquired a mature morphology similar to that which we have described previously (19).

After this period another unusual profile, which contained dense cored vesicles as well as microtubules immunoreactive for CGRP, was identified within the grafts (Fig.2). It is likely that this structure corresponds to the longitudinal profiles of regenerated CGRP-labeled axons with varicosities along their length that we found frequently on

light microscopic examination (18-20). At this survival period, we observed occasional myelinated axons but none that were immunoreactive for CGRP.

In general, immunoreactivity for CGRP was localized in axon terminals rather than in unmyelinated axons for the first month. It was not until 4 weeks that myelinated axons labeled for CGRP appeared and they were infrequent. After 12 weeks myelinated axons immunoreactive for CGRP were more frequently encountered. The myelin sheaths of the regenerated dorsal root axons were covered neither with basal lamina nor with collagen or reticulin fibrils of the endoneurium. Therefore, it is likely that the myelin sheath of these CGRP-labeled axons is of CNS origin (12, 36, 49). Many CGRP-labeled terminals formed synaptic contacts upon dendrites or perikarya in the first 2 weeks, but CGRP-immunoreactive axoaxonic synapses were only seen after 4 weeks (Fig.3).

The number of regenerated CGRP-labeled profiles steadily increased over the first 12 weeks. Thereafter, both the number and morphology of CGRP-immunoreactive axons and terminals remained unchanged (Fig. 4). At 48 weeks, the longest survival period examined by electron microscopy, regenerated CGRP-labeled axons and terminals were clearly identified within the transplants and large numbers of unlabeled synaptic terminals were recognized (Fig. 5a,b). These results suggest that regenerated dorsal root axons and intrinsic axons of transplant origin permanently innervate the spinal cord transplants.

Stereological analysis of transplant neuropil

Composition of neuropil (Table 1, Fig.6). At 2 weeks myelinated axons occupied less than 1% of the area fraction of the transplant regions examined, at 4 weeks approximately 5%, and after 12 weeks nearly 10%. Unmyelinated axons occupied about 3% of the area fraction for the first 12 weeks and increased to 9% at 24 weeks. Terminals constituted 16% of the area fraction at 2 weeks and increased to 35% by 36 weeks.

Fig.6 is a histogram showing the area occupied by each structure after correction for the distribution area of CGRP-labeled axons (data from 18). This shows the striking increase in distribution area of CGRP-labeled axons between 4 and 12 weeks. The area occupied by most structures also showed a significant difference between the first 4 weeks and 12-48 weeks after grafting, indicating continued development during the first 12 weeks after transplantation. The maximum percentage occupied by axon terminals, which include regenerated CGRP-labeled terminals, was reached at 36 weeks.

CGRP-labeled axons and terminals (Table 2). The stereological analysis of axons and terminals immunoreactive for CGRP reveals the percentage of the area fraction of the total transplant neuropil that is occupied by components of this subset of regenerated dorsal root axons. At 4 weeks myelinated axons labeled for CGRP made up approximately 1% of the area fraction occupied by all the myelinated axons. This percentage increased to 4.5% by 12 weeks and did not change significantly thereafter. At 2 weeks axons immunoreactive for CGRP represented about 20% of the area fraction occupied by unmyelinated axons and approximately 40% of the area fraction occupied by terminals. These percentages showed no further change for up to 48 weeks after

transplantation. Therefore, considering the composition of the transplant neuropil (Fig.6), the area occupied by regenerated CGRP-labeled axons and terminals appears to increase up to 12 weeks and then persist unchanged at 48 weeks.

Synaptic density (Table 3). The number of synapses per 100 μm^2 in 1-week-old transplants was 0.69. This total includes 0.45 / 100 μm^2 that are immunoreactive for CGRP and 0.24 / 100 μm^2 that are unlabeled. By 2 weeks the synaptic density had more than doubled, principally due to an increase of CGRP-unlabeled synaptic terminals. Because of the limited numbers of synapses encountered in the early weeks after transplantation, changes in synaptic density could only be compared statistically in groups that survived for 4 weeks and longer. The total synaptic density increased between 4 and 12 weeks when it stabilized. The density of synapses in which the presynaptic endings were labeled for CGRP increased until 24 weeks, whereas the density of unlabeled terminals increased up to 12 weeks. Therefore the density of regenerated CGRP-labeled synaptic terminals appears to reach a plateau later than that of the unlabeled presynaptic terminals.

CGRP-labeled synaptic vesicles (Table 2). In contrast with our previous study in which approximately 85% of labeled axon terminals in transplants contained only spherical vesicles (19, 20), in the present study approximately 55% of labeled axon terminals contained both spherical and dense cored vesicles, 45% only spherical vesicles, and approximately 1% pleomorphic vesicles. We attribute this difference to the greater sensitivity of the ABC procedure that we used in the present

study to demonstrate CGRP labeling compared with the PAP method used previously. Nearly 80% of the labeled axon terminals that contained both spherical and dense cored vesicles, 5% of those that contained spherical vesicles, and none of those with pleomorphic vesicles were immunoreactive for CGRP. The percentages of immunoreactive axon terminals did not change significantly at different survival times.

Morphometric analysis of myelinated axons and synaptic terminals

Development of myelinated axons (Table 4). The morphometric analysis of myelinated axon development included both CGRP-labeled and CGRP-unlabeled processes. The mean diameter of myelinated axons was below 1 mm at all survival periods. Most of those that were regenerated dorsal root axons would therefore be classified as A - δ fibers, as are the majority of CGRP-labeled myelinated dorsal root axons found in the superficial dorsal horn of normal adult spinal cord (5, 19, 30). The diameter of myelinated axons labeled for CGRP increased slowly up to 36 weeks, when it became similar to that of unlabeled myelinated axons. Because the axonal diameter and the thickness of internal mesaxon did not change significantly, the increase was mainly due to thickening of the myelin sheath. The myelin index was 0.71 at 4 weeks after transplantation, decreased significantly to 0.66 at 12 weeks, indicating increased thickness of myelin sheath, and then persisted unchanged.

Synaptic terminals (Table 5). The mean area of regenerated CGRP-containing synaptic terminals was significantly larger than that of

unlabeled terminals at all survival times. The area of CGRP-labeled synaptic terminals did not change, whereas that of unlabeled synaptic terminals increased until 24 weeks.

Synaptic contacts (Table 6). The average length of the synaptic contacts formed by CGRP-immunoreactive terminals was significantly greater than that of unlabeled terminals. It did not change significantly after 4 weeks in labeled terminals, but the length of synaptic contacts formed by CGRP-unlabeled terminals continued to increase slowly over the entire period studied.

75-80% of CGRP-labeled terminals and over 90% of CGRP-unlabeled terminals formed synaptic contacts with one neuronal profile (simple type). However, 20-25% of CGRP-labeled terminals contacted more than one profile (complex type). This percentage was considerably greater than that of the complex synaptic contacts formed by CGRP-unlabeled terminals (<10%) and therefore the ratios of labeled and unlabeled axon terminals that formed simple or complex synaptic contacts were very different. Because the proportion between simple and complex synaptic terminals did not change during the survey period, the multisynaptic index also did not change.

Postsynaptic structures (Table 7). The proportions of axodendritic, axosomatic, and axoaxonic synaptic contacts did not change significantly from 4 weeks to 48 weeks. Approximately 85% of CGRP-labeled synaptic terminals contacted dendrites, 5% contacted perikarya, and 10% contacted axons, whereas over 95% of CGRP-unlabeled synaptic terminals contacted dendrites and nearly 5% contacted either perikarya

or axons. The percentage of CGRP-labeled synaptic terminals with axoaxonic synapses was therefore far greater than that of CGRP-unlabeled synaptic terminals (19).

DISCUSSION

The results of the present study demonstrate that regenerated dorsal root axons have established synapses with transplanted embryonic spinal cord neurons by 1 week after surgery, that the synaptic density of the regenerated terminals increases for 24 weeks, and that these synapses remain at 48 weeks, the longest survival time studied. These results, therefore, suggest that the reinnervation is permanent. In general, most of the parameters measured in the present study show significant differences between the first 12 weeks and later survival periods, indicating that the dynamic phase of dorsal root regeneration occurs during the first few months and that the regrowth of DRG axons thereafter becomes stabilized.

Development of transplant neuropil

Except for differentiated myelin-free regions that resemble substantia gelatinosa (23, 38, 39), the lamination patterns of spinal cord transplants are not obvious and the overall structure of transplants does not resemble that of normal spinal cord (19, 39). Because the same regions cannot be identified and compared in transplants, differences in composition of transplant neuropil at different survival periods must be interpreted with caution. Nevertheless our results suggest that the maturation of transplant neuropil develops in parallel with the maturation of the regenerated dorsal roots. The area fraction occupied by most of the structures that we analyzed stereologically varies little among the transplant groups, indicating that, although the total

transplant neuropil increases, the composition of neuropil does not differ greatly at any time. The area occupied by each component of the neuropil, however, increases in parallel for 12 weeks and then does not change further. The regeneration of dorsal root axons into transplants occurs over a similar period of time (18) and therefore correlates with the development of the transplant neuropil. The prolonged ingrowth of dorsal root axons within spinal cord transplants compared with the time course of normal dorsal root development (8) might be related to the time course of development of transplant neuropil. The mechanism by which transplants affect the slow development and the maintenance of dorsal root regeneration is unclear (18). Determining the development of the molecular composition of the extracellular environment in fetal spinal cord transplants may clarify this mechanism.

Regeneration of dorsal root axons

The present results using electron microscopy identify regenerated CGRP-immunoreactive presynaptic terminals within transplants at one week following dorsal root transection. Synapses have also been observed in grafts of fetal thalamic tissue at 7 days after transplantation, the earliest time reported (34). It is likely that some synapses may have formed even earlier. We have observed varicosities along regenerated CGRP-labeled axons with light microscopy that suggest that synapses are present at 1 and 4 days after axotomy (18) and electron microscopic studies of embryonic amphibian spinal cord show that synapses develop within a few hours after axon ingrowth (10, 11, 22, 48). The synapses formed by regenerated axons appear to become morphologically mature

between one and two weeks after axotomy. The synaptic terminals of both CGRP-labeled and unlabeled axons appear immature in 1-week-old transplants, but by 2 weeks their morphology is similar to that of the synaptic endings which we have described previously in transplants after 1 month (19).

Two processes are likely to be involved in the formation of synapses by regenerated axons (24): regenerative ingrowth and terminal formation or arborization (reactive reinnervation). We observed large growth cone-like structures immunoreactive for CGRP only in 1-week-old transplants, and the most rapid phase of synaptogenesis by regenerated DRG axons occurred during the first 2 weeks. This phase of synapse formation is likely to be due to regenerative ingrowth. The completion of synaptic replacement continued over a considerably longer interval, since the synaptic density of regenerated CGRP-labeled terminals continued to increase until 24 weeks. The time course of synaptogenesis of regenerated dorsal root axons is similar to that of reactive synaptogenesis found in the adult rat dentate molecular layer following unilateral entorhinal ablation (13, 14, 28, 29, 33). We have previously observed that the synaptic density of regions of 4- to 12-week-old transplants that are richly innervated by regenerated dorsal root axons remains less than that found in lamina I of normal adult spinal cord (19). Reactive synaptogenesis in response to vacant synaptic space (7, 37) may therefore also contribute to synapse formation in the transplants.

CGRP-containing dorsal root axons that have regenerated into transplants do not become myelinated until 4 weeks after axotomy, but the area that they occupy then increases for 12 weeks, and remains stable thereafter. The maturation of this subset of regenerated dorsal

root axons is therefore similar to that of others. The myelin sheaths of these axons appear to derive from oligodendrocytes rather than Schwann cells because they are covered neither by basal lamina nor by collagen or reticulin fibrils of the endoneurium (12, 36, 49). Previous observations have suggested that Schwann cells are not present in the parenchyma of embryonic spinal cord transplants. We found laminin, which is synthesized in culture by Schwann cells regardless of whether they contact axons (6), within mature transplants only in association with blood vessels (25). The present results therefore suggest that, after the cut myelinated dorsal root axons degenerate and lose their myelin sheaths, the axons regenerate into the transplants as unmyelinated axons which become remyelinated by myelin sheaths of oligodendrocytic origin. Similarly regenerated retinal axons in goldfish optic tectum are also remyelinated over a prolonged period (31).

The results of this study indicate that regenerated dorsal root axons and axon terminals are permanently retained within embryonic spinal cord transplants. The transected axons of retinal ganglion cells that regenerate along a transplanted segment of peripheral nerve also retain their capacity to form well-differentiated synapses that persist permanently in normal and abnormal targets (1, 49). Whether embryonic spinal cord transplants enable the severed dorsal root axons to restore functional neural circuits with host spinal cord remains to be determined.

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LEGEND FOR FIGURES

Figure 1. Electron micrographs from 1-week-old transplants. Bar=1 μm. **a**: A CGRP-immunoreactive complex terminal (asterisk) containing spherical and dense-cored vesicles makes asymmetric synaptic contacts (arrows) upon dendrite (D) and soma (S). An immature profile (star) including a few spherical vesicles immunoreactive for CGRP makes a poorly differentiated synaptic contact (arrowhead) upon a dendrite (D). **b**: A growth cone-like structure filled with mitochondria, microtubules, and CGRP-labeled synaptic vesicles contacts a perikaryon (arrowheads).

Figure 2. Electron micrographs from 2-week-old transplants. Terminal formation by unmyelinated axon that contains CGRP-labeled microtubules (arrowheads) and synaptic vesicles and makes a synaptic contact (arrow) upon a dendrite (D) Bar=1 μm.

Figure 3. A CGRP-labeled presynaptic terminal synapses (arrow) with CGRP-labeled axon (A) within a 36-week-old transplant. Bar=1 μm.

Figure 4. A low magnification electron micrograph shows a densely CGRP-innervated region from a 36-week-old transplant. Nine presynaptic terminals immunoreactive for CGRP make asymmetric synaptic contacts (arrows) upon dendrites (D). Dense glial processes (G) are also present. Bar=2 μm.

Figure 5. Electron micrographs from 48-week-old transplants. Bar=1 μm. **a**: A typical CGRP-immunoreactive complex presynaptic terminal

forms synapses (arrows) with dendritic profiles. **b**: Terminal formation by unmyelinated axon containing CGRP-labeled microtubules (arrowheads) and synaptic vesicles that makes a synaptic contact upon a dendrite.

Figure 6. Composition of neuropil. Histogram summarizing stereological data corrected by distribution area of CGRP-immunoreactive axons (data from Itoh et al.'92a). Bars are divided according to the area occupied by myelinated and unmyelinated axons, terminals, perikarya and dendrites, and glia. Significant differences are as follows:

Myelinated axons: 2, 4 < 36, 24, 12, 48

Unmyelinated axons: 2, 4 < 48, 36, 12, 24

Terminals: 2, 4 < 24, 12 < 48 <36

Cell bodies & dendrites: 2 < 4 < 36, 48, 24, 12

Glia: 2, 4, 24, 12, 36, 48

Overall significance is determined by one-way ANOVA ($p<0.05$) and Duncan's multiple range test ($p<0.05$).

TABLE 1. Development of Transplant Neuropil (% of Area)

Postgraft Interval (wks)	N	Myelinated Axons	Unmyelinated Axons	Terminals	Perikarya & Dendrites	Non-neuronal structures (% of Glia)
2 (A)	4	0.64 ± 0.35	3.67 ± 0.46	15.50 ± 2.60	40.78 ± 1.42	39.41 ± 1.84 (99.57 ± 0.37)
4 (B)	4	4.78 ± 0.97	3.22 ± 0.44	21.27 ± 1.25	34.65 ± 2.24	36.08 ± 2.55 (99.14 ± 0.12)
12 (C)	4	9.11 ± 0.96	7.52 ± 0.89	23.73 ± 1.06	38.21 ± 1.83	21.43 ± 2.68 (96.99 ± 0.44)
24 (D)	4	9.14 ± 1.09	9.89 ± 1.36	25.83 ± 1.72	34.18 ± 1.61	20.96 ± 3.65 (95.87 ± 1.25)
36 (E)	4	6.76 ± 0.25	7.04 ± 0.45	34.64 ± 3.73	29.49 ± 0.59	22.07 ± 1.71 (98.15 ± 0.26)
48 (F)	4	10.33 ± 0.56	6.47 ± 0.71	27.20 ± 0.59	30.94 ± 0.80	25.06 ± 1.00 (98.46 ± 0.62)
Significance[1]		A,B,E,C,D,F	B,A,F,E,C,D	A,B,C,D,F,E	E,F,D,B,C,A	D, C, E, F, B, A

Values are mean ± S.E.M.

[1] Groups are ranked in ascending order according to means. Those groups underlined by the same line are not significantly different from one another. Overall significance determined by the Kruskal-Wallis one-way ANOVA ($P<0.05$) and individual posthoc multiple comparisons ($P<0.05$) (Seigel,'90).

TABLE 2. Development of % CGRP-Labeling of Axons and Terminals

Postgraft Interval (wks)		N	Myelinated Axons	Unmyelinated Axons	Terminals	Spherical Vesicles (% of Area)	Spherical & Dense cored Vesicles (% of Area)	Pleomorphic Vesicles (% of Area)
2	(A)	4	-	21.34 ± 4.28	44.61 ± 6.09	3.14 ± 0.56	83.54 ± 3.54	-
						(46.61 ± 4.98)	(50.86 ± 5.52)	(1.12 ± 0.57)
4	(B)	4	1.12 ± 0.97	32.77 ± 6.90	42.20 ± 2.94	3.67 ± 1.50	70.61 ± 2.35	-
						(41.34 ± 2.91)	(57.50 ± 3.00)	(1.16 ± 0.13)
12	(C)	4	4.51 ± 0.78	23.53 ± 3.67	43.18 ± 3.40	3.21 ± 0.61	75.75 ± 2.66	-
						(44.25 ± 2.66)	(54.99 ± 2.90)	(1.24 ± 0.17)
24	(D)	4	4.11 ± 0.70	21.27 ± 3.15	37.55 ± 3.59	4.33 ± 1.18	67.98 ± 5.26	-
						(46.58 ± 1.99)	(51.77 ± 2.35)	(1.66 ± 0.40)
36	(E)	4	7.43 ± 2.47	27.49 ± 2.43	51.44 ± 5.52	6.75 ± 1.22	75.51 ± 3.73	-
						(34.60 ± 4.37)	(56.63 ± 4.52)	(0.94 ± 0.14)
48	(F)	4	5.77 ± 0.32	28.35 ± 3.79	48.93 ± 1.86	10.00 ± 1.44	83.27 ± 1.30	-
						(45.90 ± 2.78)	(53.11 ± 2.80)	(0.99 ± 0.06)
Significance[1]			B<D,C,F,E	NSD	NSD	NSD	NSD	NSD

Values are mean ± S.E.M.

[1]Groups are ranked in ascending order according to means. Those groups underlined by the same line are not significantly different from one another. Those groups indicated by < are significantly different from one another. Overall significance determined by the Kruskal-Wallis one-way ANOVA ($P<0.05$) (Siegel,'90). NSD: No significant difference among the groups.

TABLE 3. Development of Synaptic Density[1]

Postgraft Interval (wks)	N	CGRP-labeled synapses/100μm²	CGPR-unlabeled synapses/100μm²	Total/100μm²
1[2]	4	0.45	0.24	0.69
2[3]	4	0.54	0.98	1.52
4 (A)	4	0.82 ± 0.12	1.23 ± 0.19	2.05 ± 0.26
12 (B)	4	1.17 ± 0.08	2.18 ± 0.16	3.35 ± 0.19
24 (C)	4	1.25 ± 0.08	2.44 ± 0.31	3.70 ± 0.35
36 (D)	4	1.52 ± 0.15	1.94 ± 0.11	3.46 ± 0.19
48 (E)	4	1.19 ± 0.06	2.07 ± 0.20	3.27 ± 0.24
Significance[4]		A,B,E,C,D	A<D,E,B,C	A<E,B,D,C

[1]Values are mean ± S.E.M.

[2]Data from 14 CGRP-labeled and 12 CGRP-unlabeled synaptic terminals.

[3]Data from 27 CGRP-labeled and 60 CGRP-unlabeled synaptic terminals.

[4]Groups are ranked in ascending order according to means. Those groups underlined by the same line are not significantly different from one another. Those groups indicated by < are significantly different from one another. Overall significance determined by one-way ANOVA ($P<0.05$) and individual posthoc comparisons are with the Duncan's multiple range test ($P<0.05$).

TABLE 4. Development of Myelinated Axons

Postgraft Interval (wks)	N	Myelinated Axon Diameter (μm)		Axonal Diameter (μm)		Thickness of Myelin Sheath (μm)		Internal Mesaxon (μm)		Myelin Index[1]	
		CGRP-L[2]	CGRP-UL[3]	CGRP-L	CGRP-UL	CGRP-L	CGRP-UL	CGRP-L	CGRP-UL	CGRP-L	CGRP-UL
4 (A)	4	0.689 ± 0.034	1.006 ± 0.067	0.491 ± 0.028	0.712 ± 0.050	0.049 ± 0.004	0.088 ± 0.006	0.050 ± 0.007	0.058 ± 0.007	0.713 ± 0.018	0.708 ± 0.006
12 (B)	4	0.740 ± 0.046	0.894 ± 0.031	0.489 ± 0.039	0.611 ± 0.026	0.084 ± 0.003	0.098 ± 0.002	0.042 ± 0.006	0.044 ± 0.001	0.661 ± 0.017	0.683 ± 0.006
24 (C)	4	0.878 ± 0.037	1.067 ± 0.063	0.612 ± 0.033	0.738 ± 0.044	0.087 ± 0.006	0.127 ± 0.010	0.046 ± 0.004	0.037 ± 0.003	0.697 ± 0.017	0.692 ± 0.006
36 (D)	4	0.984 ± 0.096	0.976 ± 0.006	0.653 ± 0.102	0.606 ± 0.030	0.098 ± 0.016	0.131 ± 0.009	0.068 ± 0.023	0.043 ± 0.003	0.664 ± 0.047	0.643 ± 0.012
48 (E)	4	0.919 ± 0.032	0.965 ± 0.042	0.607 ± 0.020	0.606 ± 0.030	0.112 ± 0.006	0.136 ± 0.007	0.044 ± 0.004	0.043 ± 0.005	0.661 ± 0.007	0.628 ± 0.009
Significance[4]		A,B,C,E,D	NSD	NSD	NSD	A,B,C,D,E	A,B,C,D,E	NSD	NSD	E,B,D,C,A	E,D,B,C,A

Values are mean ± S.E.M.

[1]Myelin index is calculated by axonal diameter divided by diameter of myelinated axon (Blight and Young,'90).

[2]CGRP-labeled myelinated axons.

[3]CGRP-unlabeled myelinated axons.

[4]See footnote 4, Table 3. NSD: No significant differences among the groups.

TABLE 5. Development of Synaptic Terminals

Postgraft Interval (wks)		N	Area (μm^2) CGRP-L[1]	Area (μm^2) CGRP-UL[2]
1[3]		4	1.97	0.84
2[4]		4	2.01	0.46
4	(A)	4	2.08 ± 0.13	0.74 ± 0.02
12	(B)	4	1.72 ± 0.16	0.66 ± 0.04
24	(C)	4	2.05 ± 0.13	0.81 ± 0.02
36	(D)	4	2.61 ± 0.30	0.90 ± 0.03
48	(E)	4	2.16 ± 0.27	0.79 ± 0.03
Significance[5]			NSD	B,A<E,C,D

Values are mean ± S.E.M.

[1]CGRP-labeled synaptic terminals.

[2]CGRP-unlabeled synaptic terminals.

[3]See footnote 2, Table 3.

[4]See footnote 3, Table 3.

[5]See footnote 4, Table 3. Area of CGRP-labeled synaptic terminals is significantly different from those of CGRP-unlabeled synaptic terminals. NSD: No significant difference among the groups.

TABLE 6. *Development of Synaptic Contacts*

Postgraft Interval (wks)	N	Synaptic Contact Length (μm)		CGRP-L[1]		CGRP-UL[2]		MSI[3]	
		CGRP-L	CGRP-UL	Simple (%)	Complex (%)	Simple (%)	Complex (%)	CGRP-L	CGRP-UL
1[4]	4	0.84	0.66	77.78	22.22	100.00	-	1.29	1.00
2[5]	4	1.13	0.48	80.00	20.00	98.33	1.67	1.47	1.02
4 (A)	4	0.78 ± 0.05	0.47 ± 0.01	75.00 ± 3.21	25.00 ± 3.21	94.25 ± 2.95	5.75 ± 2.95	1.32 ± 0.06	1.08 ± 0.04
12 (B)	4	0.68 ± 0.09	0.42 ± 0.03	79.39 ± 3.60	20.61 ± 3.60	97.14 ± 0.69	2.86 ± 0.69	1.33 ± 0.09	1.02 ± 0.01
24 (C)	4	0.76 ± 0.02	0.42 ± 0.01	79.62 ± 1.81	20.38 ± 1.81	95.45 ± 0.55	4.55 ± 0.55	1.25 ± 0.02	1.05 ± 0.01
36 (D)	4	0.73 ± 0.03	0.47 ± 0.01	76.08 ± 3.23	22.03 ± 4.02	91.39 ± 2.23	8.61 ± 2.23	1.33 ± 0.05	1.13 ± 0.06
48 (E)	4	0.72 ± 0.03	0.51 ± 0.03	81.06 ± 3.70	18.94 ± 3.70	92.59 ± 2.34	7.41 ± 2.34	1.26 ± 0.06	1.09 ± 0.03
Significance[6]		NSD	C,B,A,D,E	NSD	NSD	NSD	NSD	NSD	NSD

Values are mean±S.E.M.

[1]See footnote 1, Table 5.

[2]See footnote 2, Table 5.

[3]Multisynaptic index.

[4]See footnote 2, Table 3.

[5]See footnote 3, Table 3.

[6]*Statistical significance of length of synaptic contacts, MSI is determined by the protocol of footnote 4, Table 3 and that of percentage of simple and complex types by the protocol of footnote 1, Table 1.* NSD: No significant differences among the groups.

TABLE 7. Development of Postsynaptic Structures[1] (%)

Postgraft Interval (wks)	N	Axodendritic		Axosomatic		Axoaxonic	
		CGRP-L[2]	CGRP-UL[3]	CGRP-L	CGRP-UL	CGRP-L	CGRP-UL
1[4]	4	88.89	100.00	11.11	-	-	-
2[5]	4	80.00	98.36	20.00	1.64	-	-
4	4	86.46 ± 4.52	97.76 ± 0.66	5.72 ± 2.63	1.55 ± 0.78	7.82 ± 1.97	0.69 ± 0.60
12	4	86.22 ± 1.23	94.85 ± 0.90	3.35 ± 0.69	2.78 ± 1.04	10.44 ± 1.17	2.37 ± 0.68
24	4	84.59 ± 0.67	97.10 ± 1.45	3.56 ± 0.14	1.81 ± 0.94	11.85 ± 0.77	1.09 ± 0.60
36	4	86.28 ± 2.16	90.35 ± 6.39	2.58 ± 0.54	3.62 ± 2.19	11.15 ± 2.21	1.41 ± 0.78
48	4	90.71 ± 0.30	96.49 ± 0.83	4.12 ± 1.20	2.01 ± 0.71	5.17 ± 1.45	1.50 ± 1.30

Values are mean ± S.E.M.

[1]There are no significant differences among the groups.

[2]See footnote 1, Table 5.

[3]See footnote 2, Table 5.

[4]See footnote 2, Table 3.

[5]See footnote 3, Table 3.

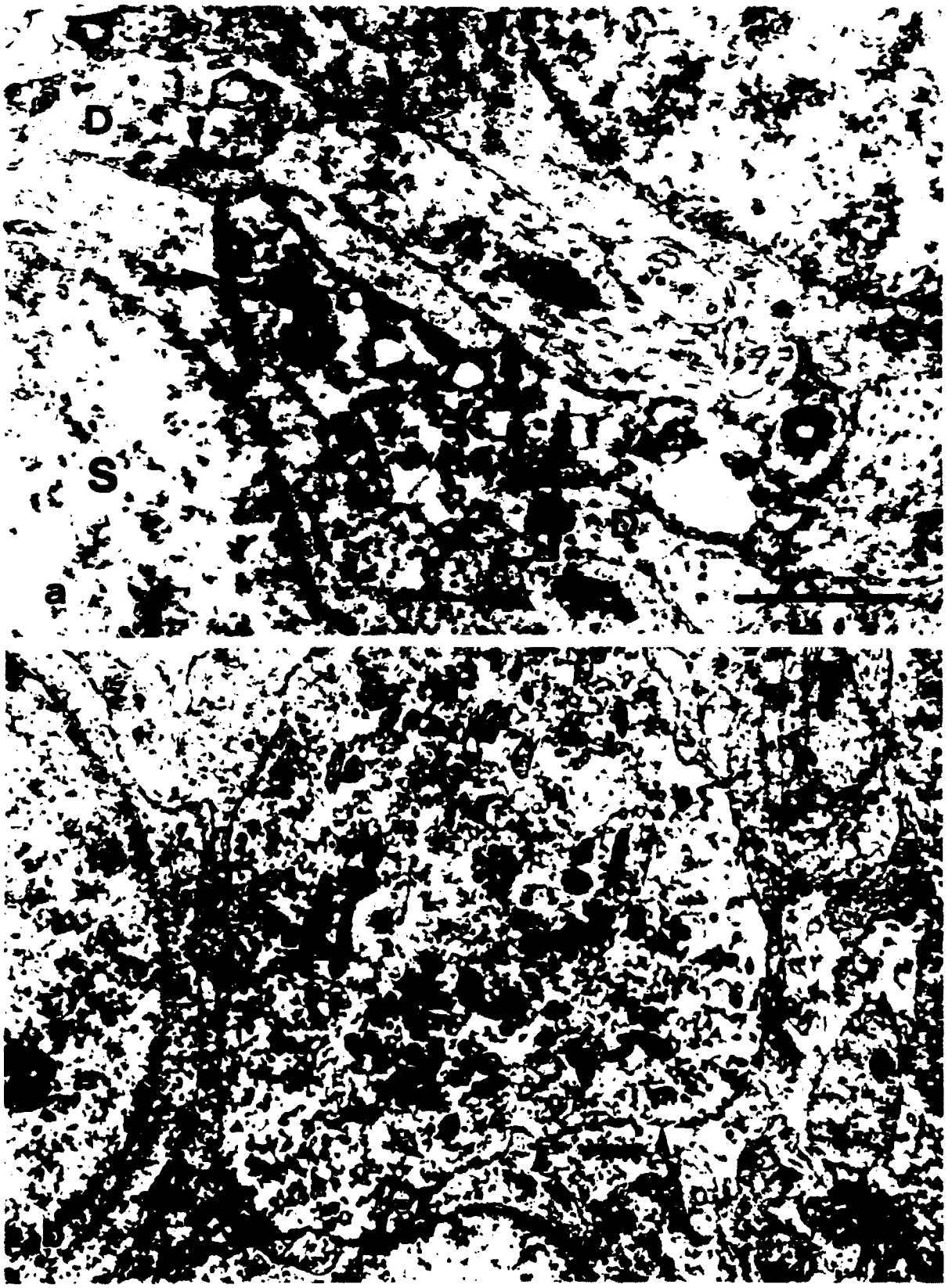

Fig 1

Fig 2

Fig 3

Fig 4

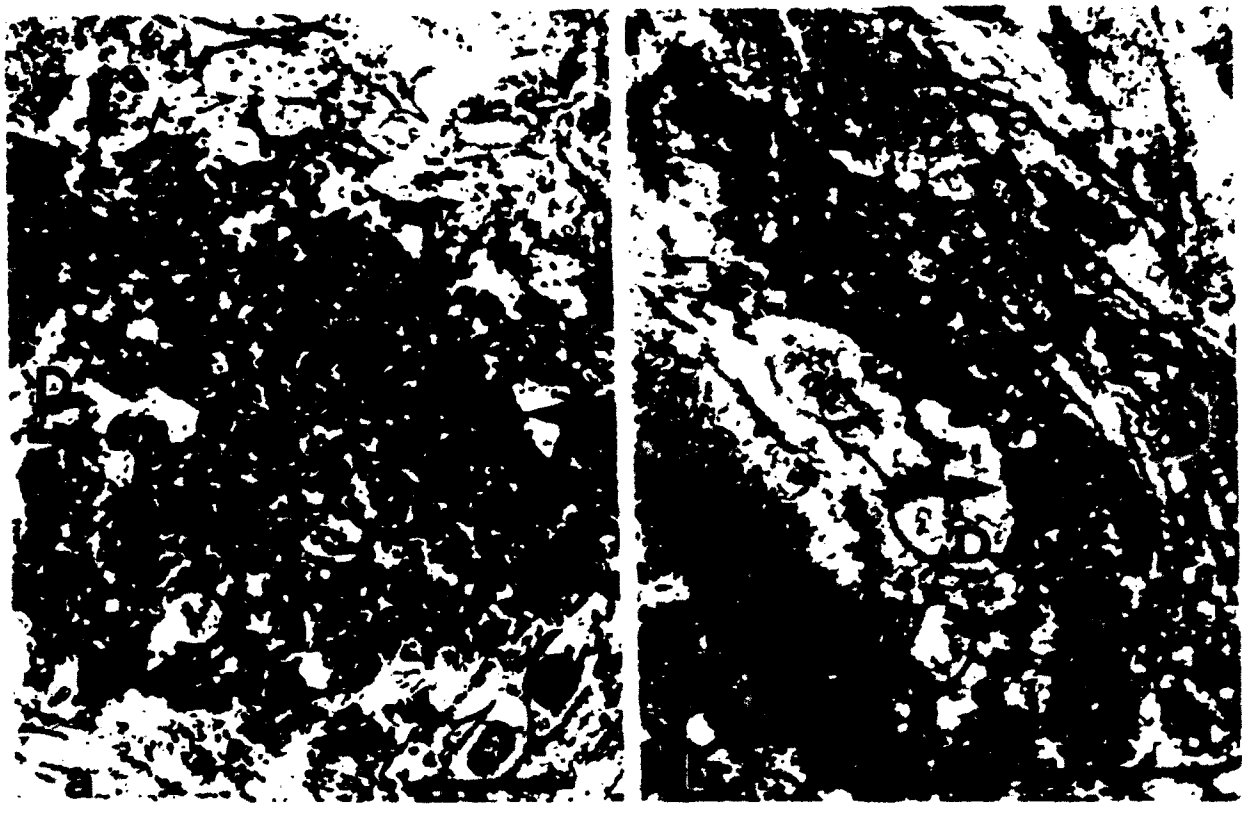

Fig. 5

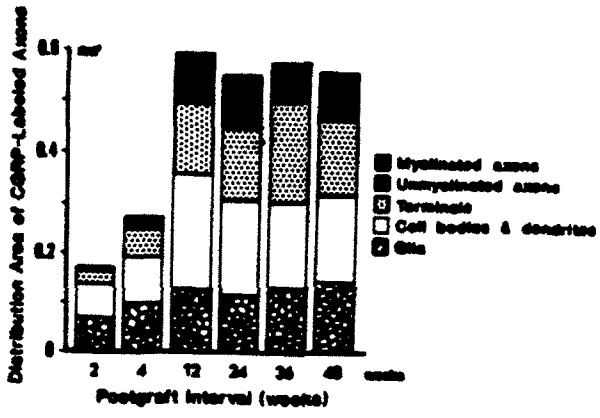
Distribution Area of CGRP-Labeled Axons
0.6
0.4
0.2
0
2
4
12
24
36
48
weeks
Postgraft Interval (weeks)
Myelinated axons
Unmyelinated axons
Terminals
Cell bodies & dendrites
Glia

Fig 6

Advances in Stereotactic and Functional Neurosurgery ... (In Press) ...

APPENDIX 5

727

Electrophysiological Responses in Fetal Spinal Cord Transplants Evoked by Regenerated Dorsal Root Axons

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Summary

Cut dorsal root axons regenerate into intraspinal transplants of fetal spinal cord (FSC) and establish synaptic connections there. The aim of the present study was to determine whether transplant neurons are driven synaptically in response to electrical stimulation of regenerated dorsal root axons. Adult Sprague-Dawley rats received FSC transplants (E14) into dorsal quadrant cavities at the lumbar enlargement. The cut L4 or L5 dorsal root stump was placed at the bottom of the lesion cavity and secured between the transplant and host spinal cord. Four to 10 weeks later the animals were prepared for electrical stimulation and recording. We stimulated regenerated dorsal roots and recorded extracellular single unit post-synaptic activities that were evoked close to the dorsal root-transplant interface. We used intracellular recording to observe several examples of monosynaptic EPSPs in transplant neurons evoked by dorsal root stimulation. These results indicate that the regenerated dorsal root axons establish functional connections with neurons within the transplants and suggest that FSC transplants can be used to reconstruct functional connections between neurons that have been interrupted by spinal cord injury.

Keywords: Embryonic spinal cord transplant; dorsal root ganglion neuron; regeneration; electrophysiology.

Introduction

The cut central axons of adult rat dorsal root ganglion (DRG) neurons do not regenerate into the spinal cord[5]. When normal spinal cord is replaced by a transplant of fetal spinal cord (FSC), cut DRG axons cross the dorsal root-transplant interface, grow within the transplant[7], and establish synapses with transplant neurons[3]. These synapses persist for over 60 weeks after grafting and resemble morphologically those formed in normal spinal cord[4]. Similarities between regenerated and normal synapses have important implications for the therapeutic potential of the transplantation technique, but

only if the regenerated synapses are functional and if the regenerated axons can activate transplanted neurons. We therefore studied whether FSC transplant neurons are driven synaptically in response to electrical stimulation of regenerated DRG axons.

Materials and Methods

Surgical Procedures

Seventeen female adult (200-300g) Sprague-Dawley rats were used as graft recipients. The rats were anesthetized with ketamine (76mg/kg), xylazine (7.6mg/kg), and acepromazine (0.6mg/kg), and the lumbar enlargement was exposed by a laminectomy of the T13 or L1 vertebra. After transection of the left L4 or L5 dorsal root, the distal portion of the root was reflected caudally. A dorsal quadrant cavity 3 mm in length was aspirated from the left side of the lumbar enlargement. FSC was then dissected from Sprague-Dawley rat pups (E14) and introduced into the cavity. The cut dorsal root stump encircled with a 10-0 suture for later identification was placed at the bottom of the lesion cavity and secured between the transplant and host spinal cord. The dural opening and the superficial wound were closed in layers.

Electrophysiology

Four to 10 weeks later transplanted animals were anesthetized with a mixture of ketamine and xylazine. All animals were ventilated with a respirator. End-tidal CO_2 and rectal temperature were continuously monitored and body temperature was maintained at 37°C.

The animals were then placed in a mechanical system designed to immobilize the vertebral column. The dorsal surface of the transplant was exposed and identified by the presence of the suture looped around the dorsal root during initial surgery. The dorsal root was exposed and prepared for stimulation with a bipolar hook electrode. This electrode was used routinely for dorsal root stimulation with constant current pulses of 0.2 msec duration. All exposed tissues were covered with warmed mineral oil.

All recordings of unitary activity in the transplants were accomplished with glass micropipette electrodes. A silver-ball monopolar recording electrode was placed at the dorsal root-transplant interface to monitor the size of incoming volleys evoked by dorsal root stimulation. Nerve stimulus strength was expressed as a multiple of the threshold intensity for the most excitable fibers in the dorsal root. This threshold was determined by slowly increasing stimulus strength to a level producing the first sign of electrical activity detectable by the silver-ball electrode.

Results

Analysis of the potentials recorded at the dorsal root - transplant interface indicated that the maximal conduction velocities of the dorsal roots were 50-60 m/sec. These values are similar to those of normal dorsal roots. The maximal conduction velocities of regenerated dorsal root axons within the transplants were 2-4 m/sec, suggesting that most of these axons are unmyelinated at the survival periods of 4 to 10 weeks.

Extracellular records were obtained from a total of 40 single units activated by electrical stimulation of dorsal roots. Consistent with previous morphological findings[2,3,7], most single unit activity was encountered within approximately 1 mm of the dorsal root - transplant interface. A train of at least 2 shocks was needed to secure reliable firing. The electrical thresholds for orthodromic firing were 5-10 times that of the most excitable fibers in the dorsal roots. Orthodromic firing latencies ranged from 4 to 6 msec (Fig.1). Based on the variance in the latency between the dorsal root stimulus and the unit spike, units were classified into two types. A histogram of the latencies of type I units showed a relatively narrow band, whereas type II showed a broad band. Units in which the response to high frequency stimulation (100 Hz) of the dorsal root was studied exhibited failures of spike initiation.

In several units, the dorsal root was stimulated with a train of high frequency stimuli (1,000 Hz, 5 msec train). Following these trains, the number of spikes evoked by single or paired dorsal root stimuli was increased and remained elevated for several minutes. These results indicate the presence of post-tetanic potentiation of primary afferent EPSPs in transplant neurons.

We obtained intracellular records from 8 units. EPSPs with monosynaptic latencies were evoked by single shock stimulation of the dorsal root (Fig.2). In one unit in which recording was exceptionally stable, the EPSP amplitude was observed to fluctuate considerably.

Discussion

The results of this study demonstrate that neurons within FSC transplants can be driven synaptically in response to electrical stimulation of regenerated DRG axons. The features of the neuronal firing observed extracellularly in response to stimulation of DRG axons support this condition. Synaptically-driven units display a variance in the latency between the onset of the dorsal root stimulus and the resulting unit spike that is greater than latencies observed for antidromic activation. This variance arises in part because of random fluctuations in the amplitude of the evoked EPSPs[4]. The latency variations that we observed in single unit activity are compatible with those expected of synaptic activation. Our data indicate the presence of 2 types of functional synaptic connections characterized by appreciable differences in the latency variance. We do not yet understand the basis for this difference, but one possibility is that these connections differ in strength.

Another feature of the unit activity we observed that reflects synaptic activation is an inability to follow high frequency stimulation of regenerated dorsal root axons. Some of the units we encountered could not follow stimulus frequencies of 100 Hz. Such failure can be related to depression of EPSPs that occurs during high frequency

activation of primary afferent fibers[1]. In the case of antidromic firing or conduction along primary afferent fibers, such failure does not occur until much higher stimulation frequencies are employed. Our finding of enhanced unit firing following a train of high frequency stimuli to the implanted dorsal root is also compatible with synaptic activity. In other primary afferent synapses, such stimuli patterns evoke post-tetanic potentiation of EPSP amplitude that can increase the probability of post-synaptic action potential firing. These extracellular indications of synaptic activity were confirmed by our finding of monosynaptic EPSPs during intracellular recording from several neurons located within the transplants. These results therefore demonstrate that the regenerated dorsal root axon synapses that we have previously observed within transplants are functional by electrophysiological criteria.

One way in which transplants can contribute to recovery of function following spinal cord injury is by serving as a substrate for the reformation of reflex arcs. A necessary component in this process is the type of functional synaptic connection we have now demonstrated. Such connections complete the afferent limb of the requisite reflex arc. Less information is available about the efferent limb of such arcs, although several possibilities are suggested by available anatomical data. One possibility is that the axons of transplant neurons project to host muscle and in this way reconstitute an interrupted segmental reflex.

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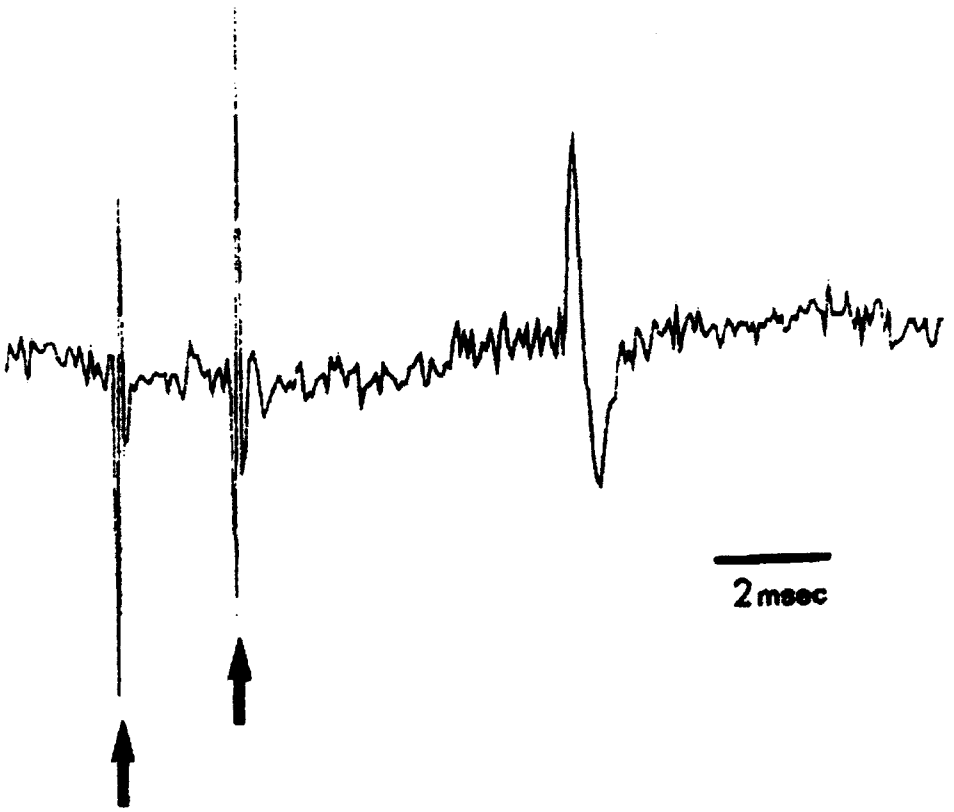
Legend for figures

Fig.1 The record illustrates an example of synaptically-driven neuronal activity in fetal spinal cord transplants following electrical stimulation of regenerated dorsal root fibers.

Fig.2 The record shows an example of an intracellular-recorded EPSP and action potential taken from fetal spinal cord transplants following dorsal root stimulation.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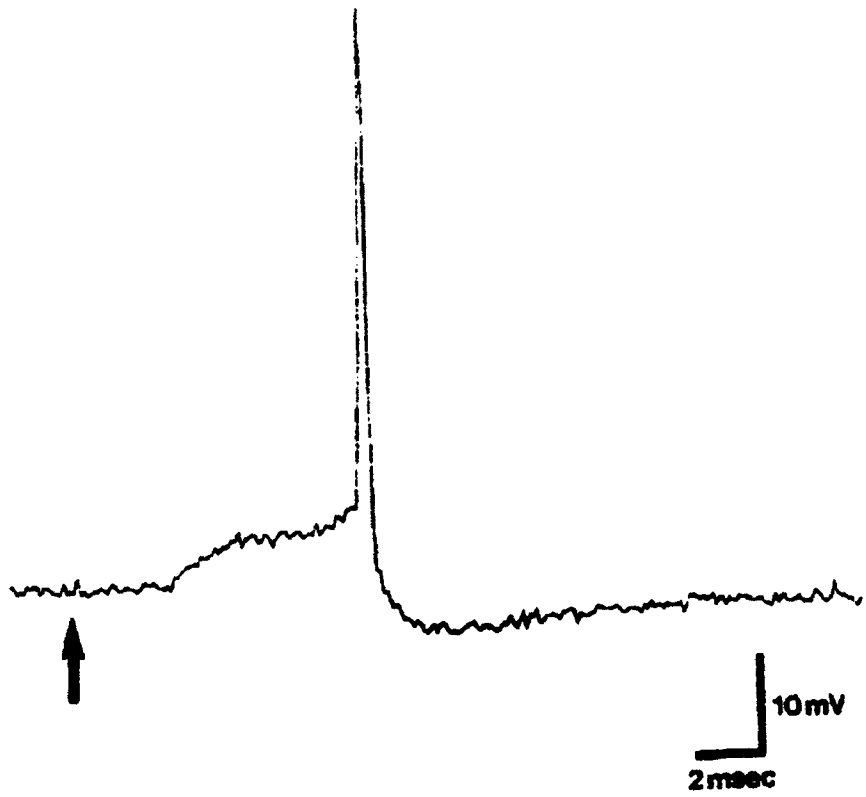
10mV
2msec

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Regeneration of Adult Dorsal Root Axons into Transplants of Dorsal or Ventral Half of Fetal Spinal Cord

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Summary

Severed dorsal root axons regenerate into the transplants of fetal spinal cord (FSC) and form synapses there. It is unknown whether the growth is specific to transplants of dorsal half FSC, a normal target of most dorsal root axons, or whether it is due to properties shared by transplants of ventral half FSC. We used calcitonin gene-related peptide immunohistochemistry to label subsets of regenerated host dorsal root axons, and morphometric analysis to compare neuronal populations within both transplants. Adult Sprague-Dawley rats received intraspinal grafts of dorsal or ventral half FSC (E14), and the L4 or L5 dorsal root was cut and juxtaposed to the grafts. Three months later sagittal sections were prepared for immunohistochemistry and Nissl-Myelin stain. Histograms of perikaryal area showed that the transplants of dorsal half FSC consisted of small neurons predominantly, whereas transplants of ventral half FSC consisted of neurons of variable sizes. Dorsal root axons regenerated into both transplants, but growth into dorsal half FSC was more robust. These results indicate that both transplants provide an environment that supports dorsal root regeneration, but that the environment provided by dorsal half FSC is more favorable. Transplants of dorsal half FSC may offer advantages for the long-term goal of repairing of damaged spinal cord circuits.

Keywords: Embryonic spinal cord transplant; dorsal root ganglion neuron; regeneration; dorsal half of fetal spinal cord

Introduction

The cut central axons of adult rat dorsal root ganglion (DRG) neurons do not penetrate the spinal cord[7]. When transplants of fetal spinal cord (FSC) are substituted for adult spinal cord, however, cut DRG axons regrow into the transplants and establish synapses there[2,3,9]. The extent to which dorsal root axons labeled for calcitonin gene-related peptide (CGRP) regenerate into the transplants persists unchanged for over 60

weeks after graft[4]. It is therefore suggestive of the proposal that FSC transplants can be used to repair damaged spinal cord circuits. It is unknown whether the growth is specific to dorsal half FSC transplants, a normal target of most DRG axons, or whether it is due to properties shared by ventral half FSC transplants.

In the present study we used CGRP immunohistochemistry to label subsets of regenerated dorsal root axons because many DRG neurons are immunoreactive for CGRP and because we have found it to be a more sensitive indicator of regenerated axons than methods that rely on axon transport or diffusion of HRP[2,3]. We also used morphometric analysis to compare neuronal populations within both transplants.

Materials and Methods

Surgery

Twenty seven female adult (200-300g) Sprague-Dawley rats were used as graft recipients. The rats were anesthetized with ketamine (76mg/kg), xylazine (7.6mg/kg), and acepromazine (0.6mg/kg), and the lumbar enlargement was exposed. After transection of the left L4/L5 dorsal root, the distal portion of the root was reflected caudally. A dorsal quadrant cavity was aspirated from the lumbar enlargement. Dorsal half (N=15) and ventral half (N=12) of spinal cord were dissected from homologous rat pups (E14) and introduced into the cavity. The cut dorsal root stump was juxtaposed to the dorsal surface of the transplants. The superficial wound were closed in layers.

CGRP Immunohistochemistry

Three months later the animals were deeply anesthetized with Nembutal (50mg/kg, i.p.) and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer pH7.4. To identify regenerated axons, every fifth cryostat section (14 μm) in sagittal plane was processed for CGRP immunohistochemistry. Sections were reacted with primary antiserum against CGRP, and immersed in biotinylated goat antirabbit IgG and avidin-biotinylated horseradish peroxidase complex. The chromagen was DAB.

To evaluate transplant morphology and the dorsal root-transplant and transplant-host spinal cord interfaces, the adjacent sections were stained with cyanine R for myelin and counterstained with cresyl violet. This procedure has been described in detail[2,3,4,9]

Quantitative analysis

The extent to which CGRP-labeled axons regenerated into transplants was measured for dorsal half FSC (N=5) and ventral half FSC (N=5) transplants. We used a point-counting stereological analysis to measure the area occupied by CGRP-labeled axons. Sagittal sections that contained labeled axons were examined under a light microscope. A micrometer 10 mm x 10 mm in size (10^4 μm^2) composed of 1-mm grid squares (Olympus, Tokyo) that was fitted in an ocular lens was used as a sampling lattice, and the number of times that CGRP-labeled axons intersected the corners of the grid squares was counted. To evaluate the arborization of CGRP-immunoreactive axons, we also measured the distribution of labeled axons in 3 sagittal sections by making composite montages that consisted of all the individual sampling lattices examined and the results were averaged. These procedures have been described in detail[3].

To compare the neuronal populations in both transplants, sagittal sections stained with cresyl violet and cyanine R were examined. Perikaryal area of cells contained in a sampling rectangle (7,450 μm^2) was measured using a Bioquant System IV (R&M Biometrics, TN). Three consecutive sections were examined per transplant. A histogram was made from these data to show the differences of cellular composition between both transplants.

The significance of the differences between both transplants was determined by Mann-Whitney two sample test ($p<0.05$).

Results

Transplants of dorsal and ventral half FSC survive in the adult host spinal cord and differentiate into patterns that are characteristic for each region. Dorsal half FSC

transplants contain regions that resemble substantia gelatinosa based on the presence of numerous small neurons and relative paucity of myelination. Ventral half FSC transplants composed of neurons of variable sizes and showed abundant myelination. Transplants are generally well-integrated with host dorsal root and spinal cord.

CGRP immunohistochemistry

Dorsal root axons immunoreactive for CGRP regenerate into every transplant examined. CGRP-labeled axons show distinctive patterns of distribution within both transplants. In dorsal half FSC, CGRP-labeled axons arborize extensively near the surface of transplants and in some portions the axons form dense bundles (Fig.1). In ventral half FSC, CGRP-immunoreactive axons extend sparsely but diffusely through the transplants and individual axons but not bundles of axons can be recognized (Fig.2).

Quantitative analysis

The point-counting stereological analysis shows the area fraction of both transplants occupied by regenerated CGRP-labeled axons. In dorsal half FSC transplants these axons occupy a mean area of 5.42×10^4 μm^2. In ventral half FSC the area occupied is approximately 40% of that in dorsal half FSC. Regenerated CGRP-labeled axons therefore occupy a significantly larger area in dorsal half FSC than in ventral half FSC (Fig.3). The mean area of CGRP-innervated regions within dorsal half FSC is not significantly different from that within ventral half FSC.

Based on the perikaryal area of neurons within the transplants, we classified the neurons into 3 groups: small-sized neurons (50 - 150 μm^2); medium-sized neurons (150 - 300 μm^2); large-sized neurons (more than 300 μm^2). Dorsal half FSC transplants include small-sized neurons significantly greater than ventral half FSC transplants, whereas ventral half FSC contain medium- and large-sized neurons greater than dorsal half FSC. These results confirm our qualitative observations.

Discussion

The pattern and extent of dorsal root ingrowth differed between transplants of dorsal half and ventral half of FSC. In dorsal half FSC regenerated axons stayed relatively close to the host dorsal root\transplant interface, arborized extensively, and were often tangled together in plexuses. In ventral half FSC, regenerated axons were distributed widely and sparsely, and grew as individual axons rather than in bundles or plexuses. These qualitative morphological observations were confirmed by our quantitative studies. Although cut dorsal roots labeled for CGRP regenerate into both transplants, the area occupied by regenerated CGRP-labeled axons within dorsal half FSC is more robust than ventral half FSC. Both transplants therefore provide an environment conductive to dorsal root regeneration, but dorsal half FSC, a normal target of most primary afferent fibers, provide additional more specific cues for growth. It is unlikely that inappropriate targets taken from ventral half FSC reproduce precisely the conditions found in dorsal half FSC. Growth into inappropriate targets is therefore consistent with the concept that the early stages of axon extension depend on molecules that are expressed generally throughout the developing nervous system[5].

Our observations that regenerated DRG axons grew more densely within dorsal half FSC than ventral half FSC suggest the presence of target-specific cues for pathfinding and target recognition that are not provided by ventral half FSC. These results are similar to those of *in vitro* studies showing that neurites of explanted fetal DRG axons grew and arborized more abundantly within co-cultured explants of spinal cord than of tectum[4]. Surface macromolecules likely to mediate the formation of specific pathways include glycoproteins that are expressed transiently by discrete populations of neurons[1].

Since there is a ventral-to-dorsal gradient of proliferation in the development of spinal cord, the ventral motor system develops earlier than the dorsal sensory system does. As development proceeds, proliferation diminishes in the ventral cord, and by E15

only the most dorsal portion of the ventricular zone remains active[6]. Since adult dorsal roots begin to regrow into the FSC transplants soon after graft[4], at the early stage of the axon elongation regeneration of adult rat dorsal roots is likely to be additionally enhanced by the precusor neurons remained in the dorsal cord.

Our results suggest that ventral half FSC as well as dorsal half FSC provide the conditions under which cut dorsal root axons can grow and survive. The conditions that constitute a permissive environment for regenerating axons are therefore relatively nonspecific, but dorsal half FSC transplants nevertheless differ in the extent to which they satisfy the requirements for growth of dorsal roots. The results suggest that dorsal half FSC transplant supply additional more specific cues for pathfinding and target recognition.

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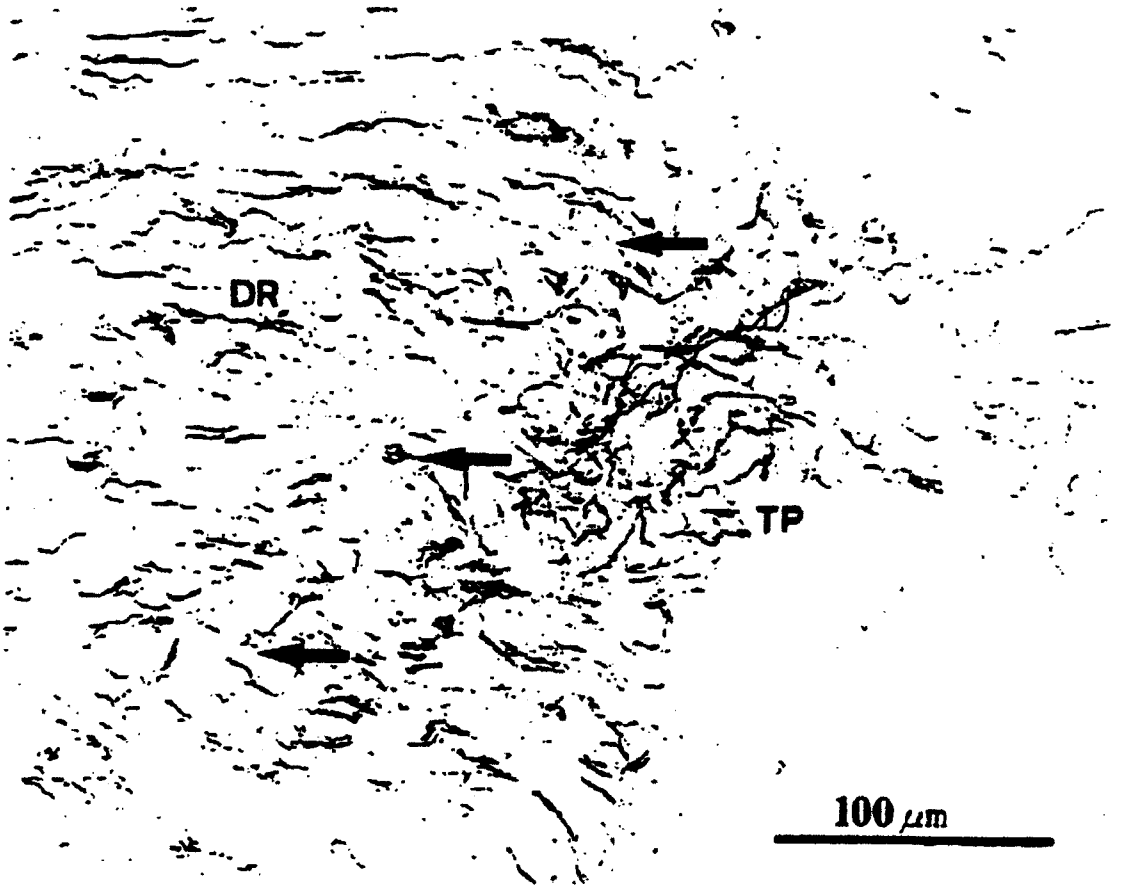
Legend for figures

Fig.1 CGRP-immunoreactive axons in the transplants of dorsal half of E14 spinal cord 3 months after transplantation. Sagittal section. Regenerated axons cross the interface (arrows) between host dorsal root (DR) and transplant (TP) and form dense plexus (*) near the interface. Interface was identified in the adjacent Nissl-stained section. Bar=100 μm.

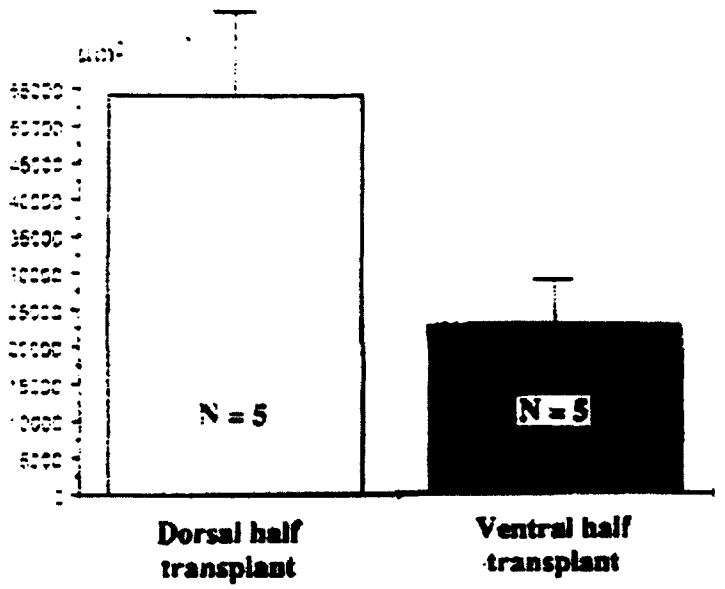
Fig.2 CGRP-immunoreactive axons in the transplants of ventral half of E14 spinal cord 3 months after transplantation. Sagittal section. Regenerated axons cross the dorsal root (DR)-transplant (TP) interface (arrows) and grow extensively within the transplant without the formation of obvious plexuses.

Fig.3 Regenerated dorsal root axons immunoreactive for CGRP occupy a significantly larger area in the transplants of dorsal half FSC than ventral half FSC. Area occupied by CGRP-labeled axons is calculated by point-counting stereological analysis (see text)


DR
TP
100 μm


DR
TP
100 μm

Comparison of Area Occupied by CGRP-Labeled Axons
(Mean ± S.E.M.)



Dr. Carla J. Shatz, Section Editor

Developmental Neuroscience

GRAFTS OF FETAL CNS TISSUE RESCUE AXOTOMIZED CLARKE'S NUCLEUS NEURONS IN ADULT AND NEONATAL OPERATES

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Running Title: Fetal CNS grafts rescue axotomized Clarke's nucleus neurons

Text pages: 20

Figures: 9

Tables: 3

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Abstract

Many conditions are thought to contribute to neuron death after axotomy, including immaturity of the cell at the time of injury, inability to re-establish or maintain target contact, and dependence upon trophic factors produced by targets. Exogenous application of neurotrophic factors and transplants of peripheral nerve and embryonic CNS tissue temporarily rescue axotomized CNS neurons, but permanent rescue may require transplants that are normal targets of the injured neurons. To determine whether this is true of adult and immature spinal cord neurons, we examined the requirements for survival of axotomized Clarke's nucleus (CN) neurons. CN in the rat consists of columns of neurons whose axons ascend ipsilaterally in the lateral funiculus to more rostral levels of the spinal cord and cerebellum. Two months after right-side hemisection of the spinal cord at the T8 segment there was a 30% loss of neurons at the L1 segment in adult operates and a 40% loss in neonates. In both age groups, neurons of all sizes were lost, but it was primarily the medium and large neurons that either died or atrophied. Transplants of embryonic spinal cord, cerebellum, and neocortex inserted into the T8 segment at the time of hemisection prevented virtually all of the cell death in both adults and neonates, but transplants of embryonic striatum were ineffective. None of the transplants prevented axotomy-induced somal atrophy. Prelabeling CN neurons by retrograde transport of fluoro-gold (FG) demonstrated that 33% of all CN neurons at L1 project to the cerebellum, 50% of these died following a T8 hemisection, but all these projection neurons were rescued by a transplant of embryonic spinal cord (the only transplant type studied under these conditions). These results suggest that the rescue of axotomized CN neurons is relatively specific for the normal target areas of these neurons, but this specificity is not absolute and may depend on the distribution and synthesis of particular neurotrophic agents.

Key words: regeneration, neuronal cell death, neurotrophic factors, transplants, axotomy, Clarke's nucleus

Many neurons pass through a developmental stage in which their survival depends on factors derived from their normal targets (reviewed in Purves and Lichtman 1985; Jacobson 1991). The survival of some neurons, such as those of the sympathetic ganglia, depends on target-derived neurotrophic factors throughout their lives (Ruit et al. 1990). For other neurons, however, the degree to which this dependence continues in the newborn and adult is unclear.

It is unknown therefore whether neuron death after axotomy in adults is due to loss of target derived factors, but several lines of evidence are consistent with this hypothesis. Injured neurons that would otherwise die following axotomy have been rescued by several different interventions. Embryonic transplants of normal target (Bregman and Reier, 1986; Haun and Cunningham 1987; Sievers et al., 1989; Tuszynski et al., 1990; Rinaman and Levitt, 1991) or peripheral nerve grafts (Bray et al., 1987; Villegas-Perez et al., 1988; Messersmith et al., 1991) have rescued axotomized CNS neurons at least temporarily and in some experiments permanently. Axotomized neurons can also be rescued by exogenous application of known factors synthesized by the normal target or nonneuronal cells along the axonal pathway such as nerve growth factor (NGF, Yip et al., 1984; Hefti, 1986; Williams et al., 1986; Kromer, 1987; Verge et al., 1989; Fischer and Bjorklund, 1991), ciliary neurotrophic factor (CNTF, Sendtner et al., 1990, 1992), interleukin-1β (IL-1β, Spranger et al., 1990), or one of the family of fibroblast growth factors (FGFs, Sievers et al., 1987; Anderson et al., 1988; Mattson et al., 1989). Target derived factors, as yet uncharacterized, have been extracted from conditioned cell culture medium that also show neurotrophic effects in neonate and adult operates (Eagleson et al., 1990, 1992).

Permanent survival after axotomy, however, may require axon growth and the reestablishment of synaptic connections with a specific target (Villegas-Perez et al., 1988; Keirstead et al., 1989) or the sprouting of surviving collateral axons (Bleier, 1969; Fry and Cowan, 1972; Sofroniew and Isacson, 1988). In newborn rats, for

example, regenerating axons of rubrospinal neurons initially invade transplants of either their normal target or of targets that they do not encounter during normal development ("inappropriate"), but later these neurons retract their axons from an inappropriate target (Bregman and Kunkel-Bagden, 1988). This implies that at least some CNS neurons require specific target derived factors to maintain their regenerated axons. Dorsal root ganglion (DRG) neurons, however, regenerate and maintain dorsal root axons in different types of intraspinal transplants, some of which are not normal targets (Itoh and Tessler, 1990).

Neuron survival following axotomy is a necessary prerequisite for axon regeneration and the restoration of normal neuronal circuitry. It is important, therefore, to understand the requirements for survival and to develop methods for providing these conditions. Factors that promote neuron survival and regeneration need to be identified. Since it continues to be technically difficult to deliver biologically active factors exogenously to injured CNS neurons, transplants of specific CNS regions provide a strategy for identifying and fulfilling the conditions necessary for neuron survival. We sought to determine whether a well defined population of intra-spinal neurons, those of Clarke's nucleus, can be rescued by transplants, whether the requirements for survival are region specific, and if the requirements for survival of neurons axotomized in adults are similar to those axotomized in newborns.

Clarke's nucleus (CN) forms bilateral columns extending from C8 to L3 in the medial aspect of the rat spinal cord (Matsushita and Hosoya, 1979). Its boundaries are distinct and readily recognized. The nucleus lies subjacent to the corticospinal tract, which lies at the base of the dorsal funiculus, and laterally is outlined by large blood vessels and heavily myelinated fascicles from the medial division of the dorsal root. CN axons ascend ipsilaterally to more rostral levels of the spinal cord and cerebellum, coursing in the dorsal portions of the lateral funiculus as the dorsal

spinocerebellar tract (DSCT) (Oscarsson, 1965; Mann, 1973; Matshusita and Hosoya, 1979; Grant and Xu, 1988). Hemisection of one side of the spinal cord therefore axotomizes only the ipsilateral CN and the contralateral CN neurons can serve as an internal control for experimental studies.

Axotomy produces extensive CN cell death which is apparent by 4 weeks in neonates (Stelzner et al., 1975; Smith and Castro, 1979) and by 2 months in adults (Feringa et al., 1987). In many neural systems cell death after axotomy in neonates exceeds that which follows the same injury in adults (Prendergast and Stelzner, 1976; Bregman and Goldberger, 1983; Himes and Tessler, 1989; Xu and Martin, 1989 & 1990). Another goal of the present study was to compare cell survival in CN after axotomy in adults and neonates.

Materials and Methods

Animals

Eighty-four Sprague-Dawley rats (Zivic-Miller, Allison Park, PA) of either sex were studied. Neonates were anesthetized by hypothermia and underwent surgery on the second or third day after birth (P2-P3). Adults (60-150 days of age and weighing 200-400 grams) were anesthetized with an intraperitoneal injection consisting of xylazine (10 mg/kg), ketamine (95 mg/kg), and acepromazine maleate (0.7 mg/kg). Control animals were age-matched for both neonate and adult operates.

Transplant tissue was taken from the embryos of timed pregnant Sprague-Dawley rats. The day of insemination was designated as E0. Donor females were anesthetized with chloral hydrate (35 mg/kg, i.p. injection) and euthanized with an overdose of the same anesthetic after removal of the embryos.

Prior to perfusion all animals were deeply anesthetized with an overdose of sodium pentobarbital (75 mg/kg, i.p. injection).

Surgery and tissue preparation

The spinal cord of deeply anesthetized rats was exposed by partial dorsal laminectomy just caudal to the blood supply of the dorsal fat pad (T7-T8 spinal cord segment). The dura was opened longitudinally at the midline and a cavity 1-3 mm in length was made by aspiration from the right side of the spinal cord. The lesion completely interrupted the lateral funiculus, including the axons of the DSCT which lie in the dorsolateral funiculus (Fig. 1). Also damaged by this lesion were regions of ipsilateral gray matter, dorsal funiculus, and ventral funiculus. Segments of tissue of appropriate size and age were dissected from embryos and introduced into the cavity using methods described previously (Reier et al., 1986). The four types of tissue used for grafting were E14 thoracic spinal cord, E15 cerebellum, E14 occipital cortex, and E14 striatum. In lesion-only animals the cavity was filled with gelfoam. In adults the dura was closed with 10-0 suture. In neonatal operates the edges of the dura were reapposed but not sutured. The lesion site was covered and the muscle and skin closed in layers. Following surgery both adult and neonatal operates were maintained on heating pads until fully awake. Fully recovered pups were then returned to their mother.

Sixty to 90 days following surgery the rats were deeply anesthetized and perfused through the heart with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The L1 spinal cord segment and lesion site were removed, dehydrated and embedded in paraffin. The T7-T8 lesion segment was serially sectioned at 15 μm and stained with the Nissl-myelin stain (see below) to verify complete DSCT section ipsilateral to the lesion without damage to the contralateral DSCT. The presence of a transplant in animals that had received a transplant was also confirmed. If the lesion partially spared the ipsilateral DSCT, damaged the contralateral DSCT or if no transplant remained in the lesion cavity of

a rat that had received a transplant, the animal was not included in the quantitative analysis.

Cell counting and cell size analysis of CN neurons

All results were obtained from CN in the L1 spinal cord segment. If the animal was in one of the operate groups, the data were collected 2-3 months following surgery. The quantitative analysis of CN neurons was performed on sections stained with a Nissl-myelin stain. The paraffin embedded L1 spinal cord segment was cut serially in cross section at 10 μm. Sections were mounted on albumin coated slides and stained to demonstrate myelin sheaths with the Cyanine R method of Clark (1981) and counterstained with cresyl violet acetate.

In every tenth section the area of CN was measured, CN neurons with a visible nucleus counted, and the cell soma area measured with the Bioquant Image Analysis System attached to a Leitz Dialux microscope at a final magnification of 678x. Neurons were classified an the basis of soma area as being small ($\leq$200 μm^2), medium (>200 μm^2 and $\leq$400 μm^2) or large (>400 μm^2). Similar criteria have previously been used for CN analysis in the cat (Loewy, 1970). Corrected cell counts were obtained with the following procedure. Nuclear diameters were measured at a magnification of 1025x for a minimum of 50 nuclear profiles from both CN in each L1 segment. These data were then processed by means of the Hendry (1976) analysis as modified by Smolen et al. (1983) to obtain a factor for corrected neuron counts. Total neuron number was determined by the formula: (correction factor x raw cell counts x distance between sections) /section thickness. A paired comparison t-test was used to analyze the numerical data for side to side differences within groups for 3 variables: the area of the CN, mean cell size, and total neuron number. The analysis was performed separately for adult and neonate groups. The groups compared were normal animals (N=6), animals with T7-T8 hemisection alone (N=6),

and hemisected animals with a graft of one of the four different types of embryonic CNS transplant (N=6 for each group). In all cases, analysis of the numerical data with a one way ANOVA showed that CN of intact controls did not differ statistically from the contralateral CN of lesioned animals in any group for any of the variables measured. Therefore we used the lesion/control ratio of each animal as the basis for comparison. Overall significance of the ratio data was determined by the Kruskal-Wallis one way ANOVA ($p<0.05$). If significant differences were present, individual posthoc comparisons were made with the Wilcoxon-Mann-Whitney test corrected for multiple comparisons (Kirk, 1968). All statistical analyses were performed with the Number Cruncher Statistical System program (Dr. Jerry L. Hintze, Kaysville UT).

Retrograde labeling of CN neurons projecting to the cerebellum

To determine whether CN neurons whose axons project to the cerebellum were among those rescued by transplants, we used the fluorescent tracer Fluoro-gold (FG, Fluorochrome, Inc., Englewood, CO). After retrograde transport FG remains in the soma of adult rat neurons for at least 2 months (McBride et al., 1988). The anterior lobe of the cerebellum was exposed by a midline incision in the skin and removal of the interparietal bone with a surgical drill. The underlying transverse venous sinus and the superior cerebellar artery were preserved. Multiple bilateral injections of a 2% aqueous solution of FG (total volume 5 μl) were made through a glass pipette (tip diameter 50 μm) attached to a 10 μl Hamilton syringe. The opening in the skull was then covered with bone wax and the wound closed in layers. Four animals had no additional procedures and served as normal controls. Seventy-two hours after FG injection into the cerebellum, a hemisection cavity was made in the T7-T8 spinal cord segment of 8 additional adults and then filled either with gelfoam (N=4) or a transplant of E14 spinal cord (N=4). After a 2 month survival period, the

animals were perfused through the heart with a flush of phosphate buffered saline (PBS) followed by fixative containing 4% paraformaldehyde and 0.02% picric acid in 0.1 M phosphate buffer, pH 7.4. The lesion site, L1 spinal cord, and cerebellum were placed in 0.1M phosphate buffer containing 30% sucrose. The lesion site was studied as described above. The cerebellum and L1 spinal cord segment were sectioned at 10 μm on a cryostat and the sections mounted onto gelatin coated slides. After air drying the slides were briefly immersed in xylene, coverslipped using DPX mounting media (Fluka Chemical Co. Ronkonkoma, NY), and observed by fluorescence microscopy. Examination of the cerebellum confirmed that the anterior lobe had been labeled bilaterally and symmetrically. In the L1 spinal cord segment the number of labeled neurons in every tenth section were counted. We also stained a series of adjacent sections from L1 with the Nissl-myelin method described above and counted and measured the neurons in CN at L1. We determined the number of CN neurons at L1 that project to the cerebellum in adult rats, and the number that remain 2 months after hemisection alone or hemisection with a transplant of E14 spinal cord. Comparison of the number of Nissl-stained cells with the number of FG-positive cells demonstrated the percentage of CN neurons in L1 that project to the anterior lobe of the cerebellum. Comparison of the mean size of Nissl-stained cells to the mean size of FG-labeled cells demonstrated the size of cells in CN that project to the cerebellum. Four additional series of sections from the L1 spinal segment were used in the immunocytochemical experiments described below.

Immunocytochemistry

Because FG can be phagocytosed by macrophages and microglia after the death of retrogradely labeled neurons (Rinaman et al., 1991), we used a series of antibodies raised against cell-specific markers to confirm that FG-labeled cells

observed in CN were neurons. These antibodies were: 1) anti- glial fibrillary acidic protein (GFAP), the major protein found in glial intermediate filaments (Bignami and Dahl, 1976; Eng et al., 1971), which recognizes astrocytes (Lazarides, 1982). 2) ED-1, which recognizes an uncharacterized cytoplasmic antigen found in all cells of the rat macrophage/monocyte line (Dijkstra et al., 1985; Sminia et al., 1987). 3) OX-42, which recognizes the complement C3bi receptor expressed by neutrophils, monocytes, macrophages and microglia (Perry et al., 1985; Robinson et al. 1986; Perry and Gordon, 1987). 4) anti- microtubule associated protein 1B (MAP1B), one of the components of cross bridges between microtubules (Sato-Yoshitake et al., 1989), which recognizes neurons. The immunocytochemical methods were modifications of the procedure of Milligan et al. (1991). The antibodies and dilutions used were: 1) GFAP (Biomedical Technologies, Inc. Stoughton, MA), a polyclonal antibody diluted 1:1000. 2) ED-1 (Harlan Bioproducts for Science, Indianapolis, IN), a monoclonal antibody diluted 1:150. 3) OX-42 (Harlan Bioproducts for Science) a monoclonal antibody diluted 1:500. 4) MAP1B (A generous gift from Dr. Itzhak Fischer), a polyclonal antibody diluted 1:1000 (Safaei and Fischer, 1989; Fischer and Romano-Clarke, 1990). All antibodies were diluted in 0.1M PBS, pH 7.4 containing 2% nonfat dry milk (Blotto). Sections were incubated at 25 ^{o}C overnight in a humid chamber. After several rinses in PBS the appropriate peroxidase-conjugated secondary antibody was applied, also diluted in Blotto: for GFAP and MAP1B a goat anti-rabbit IgG (Jackson Immunoresearch Labs Inc. West Grove, PA) diluted 1:100; for ED-1 and OX-42, a goat anti-mouse IgG (Jackson Immunoresearch Labs Inc.) diluted 1:50. Specific staining was visualized by incubating the tissue with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.St. Louis, MO) in 0.05M Trizma-HCl buffer. The sections were then air dried, briefly immersed in xylene, and coverslipped with DPX mounting media. This procedure preserved much of the FG-labeling in retrogradely labeled neurons and allowed sections to be

viewed under brightfield illumination to visualize the DAB reaction product or with fluorescence microscopy to visualize FG-labeled cells.

Results

Transplant Morphology

51 of 52 animals that received grafts contained a transplant at the end of the two month postoperative survival period. One graft of E15 cerebellum failed to survive in an adult recipient and data from this animal were not included in this study. While the grafts lacked the overall structure of the CNS region from which the tissue was taken, some areas of all transplants exhibited morphology characteristic of the original transplant tissue (Fig. 2). Transplants of spinal cord contained myelin-free regions with many small neurons that resembled the substantia gelatinosa (Tessler et al., 1988; Jakeman et al., 1989). Cerebellum transplants differentiated into molecular, granular, and purkinje cell layers (Itoh and Tessler, 1990). Neocortex contained mostly small to medium-sized neurons characteristic of the superficial cortical layers (Itoh and Tessler, 1990). Striatal grafts contained scattered small and medium neurons often arranged in clusters (DiFiglia et al., 1988; Labandeira-Garcia et al., 1991). Transplants did not always fill the lesion cavity completely, particularly in neonatal operates where the graft tissue appeared to grow more slowly than the host spinal cord. Transplants of neocortex exhibited the most exuberant growth, in some cases growing larger than the lesion cavity and surrounding the unoperated side of the host spinal cord.

A variable amount of gliosis was present at the host-transplant interface. In some animals the glial scar was minimal and there was a clear area of continuity between host and graft. In others the transplant was almost completely surrounded by dense gliosis, and there were few regions of continuity with the host spinal cord. In general the presence of a glial scar was more prominent in adult hosts.

Transplants of spinal cord and neocortex were better integrated with the host spinal cord than transplants of cerebellum and striatum, which were smaller and more isolated by gliosis. Within an experimental group (i.e. spinal cord, cerebellum, neocortex, or striatum grafts) neither the size nor the degree of integration of the transplant had an obvious relationship to its effectiveness in rescuing axotomized CN neurons.

Effects of Axotomy and Fetal Grafts on CN Neuron Survival

Adults (Table 1) CN in the L1 segment of normal animals consisted mostly of small and medium-sized neurons, but approximately 10% were large (>400 μm^2, Fig. 3, 5). The neuron cell size distribution of CN contralateral to hemisection alone or hemisection with transplant was very similar to normal. Two months after hemisection alone the area of the nucleus ipsilateral to the lesion decreased by 20% and the number of CN neurons decreased by 30% (Fig. 4a). The cell size distribution in CN ipsilateral to the lesion was significantly altered, with some loss of small and medium-sized neurons and almost complete absence of the largest cells (Fig. 3, 5). The mean soma area of surviving neurons in CN ipsilateral to the lesion alone was 30% smaller than that of neurons in the contralateral CN.

In animals with a hemisection and transplant of either embryonic spinal cord, cerebellum, or neocortex , the ipsilateral CN showed no significant cell loss (Fig. 3, 4a). Animals with a hemisection and embryonic striatal transplant, however, showed cell loss comparable to that seen after hemisection alone. The area of CN still decreased in animals that had transplants of spinal cord, cerebellum, or neocortex, but significantly less than after hemisection alone or after a striatal transplant. Mean soma size in all transplant groups was reduced to a similar extent as after hemisection alone (Fig. 5). Large neurons were absent and the number of medium-

sized cells reduced, but the proportion of small neurons ($\leq 200\ \mu m^2$) increased. Spinal cord or cerebellum transplants rescued all axotomized neurons. Animals that received transplants of neocortex showed a small but significant loss of the CN neurons (8%), but this was significantly less than that seen after hemisection alone or with a transplant of striatum. Transplants of spinal cord, cerebellum, and neocortex therefore rescued CN neurons from axotomy-induced cell death in adult operates, whereas transplants of striatum did not. None of the transplants prevented atrophy of the cell bodies of surviving CN neurons.

Neonates (Table 2) Hemisection alone caused a 40% neuron loss and a 40% decrease in the area of CN ipsilateral to the lesion (Fig. 3, 4b). Neurons of all sizes were lost, including almost all large cells and many medium-sized cells (Fig. 6). The cell size distribution in CN contralateral to the lesion was similar to that seen normally.

Two months following hemisection and insertion of a spinal cord, cerebellum, or neocortex transplant there was complete rescue of axotomized neurons in CN of the L1 segment. As in the adult operates transplants of striatum were ineffective (Fig. 3, 4b). In the three transplant groups that rescued CN neurons, the area of CN ipsilateral to the lesion was reduced by 20%, but remained significantly larger than after hemisection alone. None of the transplants prevented soma atrophy (Fig. 6). The cell size distribution for all transplant groups was shifted to that of the smaller neurons, and was virtually identical to that seen in adult operates.

Survival of Cerebellar Projection Neurons (Table 3)

We first examined the numbers and sizes of CN projection neurons in normal adult rats by injecting FG into the anterior lobe of the cerebellum. Two months after injection, 33% of CN neurons at L1 were FG labeled. Bilateral cerebellar injections produced symmetrical numbers of FG labeled CN neurons and labeled neurons of all sizes (Fig. 7). The mean size of FG labeled neurons in normal CN was 268 μm^2. This was 28% larger than the mean cell size of the entire population of CN neurons (Fig. 8). Small neurons (<200 μm^2) represent approximately 66% of CN neurons; of these small neurons 24% were FG labeled. Neurons between 200 and 400 μm^2 comprised 29% of the cells in normal CN and 88% were FG labeled. The largest cells (>400 μm^2) made up less than 10% of the neuronal population of CN at L1 but virtually all were FG labeled (Fig. 8). Therefore CN neurons of all sizes send axons to terminate in the cerebellum, but the greatest proportion of projection neurons is found in the larger cells.

Pre-labeling CN neurons in adult rats before a thoracic hemisection enabled us to determine the number of CN neurons projecting to the cerebellum that died following axotomy and how many of these were rescued by spinal cord transplants. CN contralateral to the lesion had similar numbers of neurons and a similar cell size distribution as unoperated normal adults. Two months after hemisection the ipsilateral CN at L1 contained 50% of the number of FG labeled neurons present in the contralateral CN, indicating a loss of half of the FG labeled neurons (Fig. 7, 8). The mean size of surviving FG labeled neurons ipsilateral to the lesion was 47% smaller than in the contralateral CN. Of the surviving CN neurons 80% were small, and 40% of these were FG labeled; 19% were medium-sized and 54% of these were labeled; less than 1% were large, and all of these were labeled.

Following a transplant of E14 spinal cord into the hemisection cavity there was no significant loss of FG labeled cells in the L1 spinal cord segment of pre-

labeled animals. The CN contralateral to the lesion had similar numbers of these neurons and a similar cell size distribution as unoperated normal adults. The labeled neurons in the ipsilateral CN were 31% smaller than those on the contralateral side. In this population of surviving CN neurons 77% were small, and 40% of these were FG labeled; 23% were medium-sized, and 75% of these were labeled; less than 1% were large and all of these were labeled (Fig. 7, 8). These results demonstrate that 50% of axotomized CN neurons survive even without a transplant and that transplants of fetal spinal cord rescue all CN neurons which project to the cerebellum but do not prevent atrophy of these cells.

Fluorogold Labeled Cells are Neurons

FG may have been transferred from dying neurons to phagocytic macrophages, microglia, or other adjacent glial cells (Rinaman et al., 1991). To confirm that surviving FG positive cells in CN were neurons, we used four immunocytochemical markers that are specific for different cell types. The L1 spinal cord segment was studied two months following hemisection alone or with a transplant of E14 spinal cord.

GFAP (Fig. 9a.) GFAP was localized to the cell bodies and processes of astrocytes distributed throughout the gray matter and white matter of the normal L1 segment (Murray et al., 1990). Two months following hemisection with or without a spinal cord transplant, the distribution of GFAP immunoreactivity was similar in CN contralateral and ipsilateral to the lesion. Following hemisection alone or in combination with an E14 spinal cord transplant, FG positive cells within CN were GFAP negative, indicating that they were not astrocytes.

ED-1 (Fig. 9b.) ED-1 positive cells were present next to blood vessels throughout the normal L1 segment (Milligan et al., 1991). Two months following hemisection with or without a spinal cord transplant, large numbers of ED-1 positive

cells appeared in regions of the white matter ipsilateral to the lesion that contained degenerating axons. The FG positive cells within CN were ED-1 negative, indicating that they were not macrophages or monocytes.

OX-42 (Fig. 9c.) OX-42 positive cells were scattered throughout the normal L1 segment (Milligan et al., 1991). Two months following hemisection with or without an E14 spinal cord transplant, OX-42 staining cells were present in the same regions of the spinal cord that showed ED-1 immunoreactivity. The FG positive cells within CN were OX-42 negative, indicating they were not microglia.

MAP-1B (Fig. 9d.) MAP-1B was seen primarily in the cell bodies of larger neurons in the normal L1 segment. The nucleus of these neurons remained unstained, allowing the detection of FG labeling. Two months following hemisection with or without an E14 spinal cord transplant, cells in CN that were FG positive were also MAP-1B positive, identifying them as neurons.

The results of these experiments using immunocytochemical markers confirm that the FG positive cells in CN are neurons that sent processes to the cerebellum and survived axotomy.

Discussion

The results of this study show that a mid-thoracic hemisection in adult or neonate rats causes 30-40% of CN neurons in the L1 spinal cord segment to die, that transplants of some but not all regions of the fetal CNS prevent this axotomy-induced cell death, and that neurons whose axons project to the cerebellum are among those that are rescued.

Some Axotomized Neurons are Rescued by Transplants

Transplants of embryonic spinal cord, cerebellum, or neocortex prevented

axotomy-induced cell death in both adult and neonates whereas transplants of embryonic striatum did not. This rescue occurred regardless of the size of the transplant or how well it was integrated with the host spinal cord. We therefore hypothesize that the effective transplants release at least one neurotrophic factor which is relatively specific for CN neurons and has a restricted distribution within the CNS that includes CN target regions. Other injured CNS neurons can also be rescued by grafts of their normal target or factors derived from their normal target. Developing dorsal lateral geniculate nucleus (dLGN) neurons, for example, were temporarily rescued by grafts of their normal target but not an inappropriate target (Haun and Cunningham 1984, 1987). Long-term rescue of dLGN neurons in adults followed short-term infusion of a factor derived from their normal target, implying that even transient exposure can effect permanent rescue (Eagleson et al., 1992). Transplants of their normal target, but not an inappropriate target, also ensured long-term survival of rubrospinal neurons injured during development (Bregman and Reier, 1986; Bregman and Kunkel-Bagden, 1988).

This specific relationship between CN neurons and their normal target is not absolute, since CN neurons also survived if embryonic neocortex, an inappropriate target, was used as the transplant. This region of the CNS must share some property with embryonic spinal cord and cerebellum which is absent from striatum and contributes to survival of injured CN neurons. This feature could be the production of one or more neurotrophic factor(s). One candidate molecule is neurotrophin-3 (NT-3), a member of the nerve growth factor (NGF) family that supports the survival of embryonic DRG neurons and other cell types *in vitro* (Maisonpierre et al., 1990a; Vogel and Davies 1991). Consistent with this idea is the observation that high levels of mRNA for NT-3 have been found in the developing cerebellum, neocortex, and spinal cord of approximately the same age as the embryonic tissue that we harvested for transplantation, whereas the embryonic striatum contained very low

levels (Maisonpierre et al., 1990b). The regional production of such neurotrophic molecules during development could contribute to neuron survival and/or appropriately directed axon growth of particular neuron populations. Very few *in vivo* models have been established to study the action of identified neurotrophic factors in the CNS. Transplantation of regions particularly rich in certain neurotrophic factors following lesion may provide initial insights into the regionally specific operation of these agents.

Transplants of embryonic CNS tissue may produce long-term rescue of axotomized neurons by serving as a target in which regenerating axons form synapses or as a bridge to a target in more rostral regions of the host CNS. Because the mature CNS is not permissive to long distance axon growth (Schnell and Schwab, 1990; Savio and Schwab, 1990), axotomized CN neurons in adult operates are unlikely to have regenerated their axons through the transplant and into rostral host spinal cord and cerebellum. Their axons are more likely to have ended in the transplant or in host spinal cord caudal to the transplant. Axons from adult host CNS neurons grow only short distances into intraspinal transplants and most of the perikarya of these neurons are located within a few millimeters of the grafts (Jakeman and Reier, 1991). The cut dorsal roots of adult rats, however, form synapses with neurons in E14 spinal cord transplants although the intraspinal transplants are distant from the perikarya of the regenerating axons (Itoh and Tessler, 1990; Houle et al., 1992; Reier et al., 1992; Tessler et al., 1992). Because the environment of the developing CNS is more conducive to long distance axon growth than that of the adult CNS, axons of CN neurons in neonates may traverse a transplant and rostral host spinal cord to reach their normal targets. In the newborn rat, regenerating or late growing axons of corticospinal neurons and serotonergic and other brainstem neurons grow through fetal CNS transplants and into host spinal cord many segments caudal to the transplant (Bregman 1987;

Bregman et al., 1989; Bregman and Bernstein-Goral, 1991).

We observed that transplants rescued axotomized CN neurons in both adults and neonates even when the transplants were poorly integrated with host spinal cord. In these cases, transplant - host connections or a bridging effect are unlikely, suggesting that neuron survival does not require direct contact with transplant neurons, but release of diffusible substance(s) from the graft (cf. Eagleson et al., 1992).

Some Axotomized Neurons Survive Without a Transplant

Our present results with FG labeling show that some axotomized CN neurons survive for long periods even without a transplant (see also Himes and Tessler, 1989). Why some of these neurons survive axotomy whereas others die is not clear. One likely possibility is that a subset of CN neurons have axon collaterals proximal to the lesion which contribute to survival by continuously furnishing appropriate trophic support. This mechanism has been proposed for other types of CNS neurons (Bleier, 1969; Fry and Cowan, 1972; Sofroniew and Isacson, 1988). Evidence concerning the presence of collateral axons in the vicinity of the cell body of CN neurons is conflicting (Boehme, 1968; Rethelyi, 1968; Randic et al., 1981). However, in the cat, CN neurons send collateral axons into rostral levels of the spinal cord (Liu, 1955). Several other lesion studies are consistent with the idea that CN collateral axons develop as the neurons mature and that these leave the DSCT to terminate in the spinal cord at more rostral levels. Hemicerebellectomy in the newborn rat, for example, causes extensive cell atrophy and dendritic alterations, but the same lesion in 3 week old animals causes no detectable changes (Smith and Castro, 1979). In 10 week old kittens lesions close to the cell bodies of CN neurons cause more extensive retrograde cell changes than more distal lesions (Loewy and Schader, 1977), and in the adult cat hemicerebellectomy causes no retrograde changes (Ha and Liu 1968).

Neonatal lesions may cause collateral axons to be maintained that would normally be retracted after the principal axon establishes contact with its normal target. Neurons in the developing visual cortex, for example, emit axons that initially descend caudal to their normal targets in the pons but are later retracted after collateral axons have contacted permanent targets in rostral and lateral basilar pons (O'Leary and Terashima, 1988). In addition it is possible that some axotomized neurons survive due to non-specific factors synthesized at the lesion site or in damaged fiber tracts. Prime candidates that are produced following lesions are CNTF, bFGF, and TGF-β (Nieto-Sampedro et al., 1983; Frautschy et al., 1991; Chalazonitis et al., 1992; Gomez-Pinilla et al., 1992; Lindholm et al., 1992), and neurotrophins (Ceccatelli et al., 1991; Yoshida and Gage, 1991; Lindholm et al., 1992). These and other factors mediate complex interactions among different types of cells that may be important in supplying trophic support to injured neurons (Bandtlow et al., 1990; Spranger et al., 1990; Engele and Bohn, 1991; Matsuoka et al., 1991; Petroski et al., 1991; Araujo and Cotman, 1992; Lindholm et al., 1992; Rudge et al., 1992). Furthermore, some injured neurons themselves may synthesize neurotrophins that would allow them to survive without target-derived neurotrophic support (Lindsay, 1988; Schecterson and Bothwell, 1992).

Are Developing CN Neurons More Vulnerable?

We found no significant difference in the numbers of CN neurons that die following axotomy in adults and neonates. In both groups neurons of all sizes were lost and large neurons were preferentially lost. In many systems, axotomy during the developmental period causes significantly more cell death than the same injury causes in the adult (Prendergast and Stelzner, 1976; Bregman and Goldberger, 1983; Himes and Tessler, 1989; Xu and Martin, 1989 & 1990). Other CNS neurons die in equal numbers after axotomy in neonates and adults (Chow and Dewson, 1966

Peacock and Combs, 1965; Allcutt et al., 1984). These differences may be due to the state of maturity of particular CNS pathways at the time of neonatal injury Retrograde labeling studies show that axons from at least some developing CN neurons reach the cerebellum at birth (Nunes and Sotelo, 1985). It is not known if at age P2-P3 all CN neurons that will project to the cerebellum or rostral spinal cord have sent axons as far rostral as the T7-T8 spinal segments and therefore it is unknown whether similar numbers of CN neurons were axotomized by our lesions in adults and neonates. If late growing axons from CN had not reached the level of the T7-T8 spinal cord segment before the lesion was made, they would not be injured, would not die, and, like developing corticospinal axons, they may grow around the lesion and reach their normal targets (Bregman and Goldberger, 1983). Conditions that increase the vulnerability of newborn neurons may be counterbalanced by conditions that promote survival. The survival of newborn CN neurons may, for example, be more dependent on target - derived factors than adult CN neurons, but higher levels of neurotrophic substances may be available in the developing spinal cord which could support axotomized CN neurons.

Why Do the Remaining CN Neurons Atrophy?

Axotomized CN neurons that survive in adult and neonatal operates remain atrophied or fail to develop to their normal size. The explanation for this atrophy or failure to develop is unknown. If, as suggested, a larger cell body is required to maintain the greater amounts of axoplasm in a long axon (i.e. extending to the cerebellum), then a smaller cell body may be sufficient to maintain axons that extend only to the transplant (Ramon y Cajal, 1984; Isacson and Sofroniew, 1992). Another possible mechanism for neuron atrophy is the change in afferent input following axotomy, which would imply a trophic effect of the afferent input under normal conditions. Axotomy is followed by the stripping of synapses from the cell

bodies and proximal dendrites of CN neurons (Chen et al., 1977). The synapses are replaced, but the new synapses are smaller than normal, their source is unknown, and their input may be toxic to the axotomized cell (Chen et al., 1977; Sanner and Goldberger, 1990, 1991). Finally, because the soma atrophy of NGF-sensitive DRG neurons (Verge et al., 1989) and cholinergic medial septum neurons (Hagg et al., 1989) that follows axotomy can be reversed by exogenous NGF, a third possibility is that axotomized CN neurons receive an inadequate supply of neurotrophic factors despite the presence of a transplant.

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FIGURE LEGENDS

Fig. 1 A. Illustration of the lesion paradigm used in these experiments. A hemisection cavity was aspirated at approximately the T8 spinal cord segment, interrupting the DSCT. In hemisection only animals the lesion was filled with gelfoam. If the animal was to receive a transplant, the cavity was filled with fetal tissue. B and C. Nissl-myelin stained cross sections of T8 spinal cord showing the extent of the lesion in adult (B) and neonate (C) operates; bar = 200 μm.

Fig. 2 Four examples showing sagittal sections of the caudal host(h) - transplant(tp) interface. Arrowheads indicate interface zone. The dorsal surface is toward the top of the figure. A. E14 spinal cord in adult host does not show the characteristic laminar pattern. B. E15 cerebellum in neonate host with a trilaminar structure resembling that of a cerebellar folia. C. E14 neocortex in neonate host shows the greater size of this type of graft relative to the other fetal tissues used in these experiments. D. E14 striatum in adult host showing clustering of cells within the graft. Nissl-myelin stain, bar = 200 μm

Fig. 3. A. Cross section of CN in L1 spinal cord. Area of CN is outlined in black. A. normal. B-G Representative cross sections of CN in L1 ipsilateral to: hemisection only in adult (B) and neonate (C) operates; hemisection with transplant of spinal cord, cerebellum, or neocortex in adult (D) and neonate (E) operates; hemisection with transplant of striatum in adult (F) and neonate (G) operates. Nissl-myelin stain, bar = 50 μm

Fig. 4. Cell survival in CN at L1 as represented by the ratio of the neurons on the operated side / neurons on the control side. A. Adult operates. B. Neonate operates. Asterisk (*) indicates that the ipsilateral CN of hemisection only (HX) and hemisection with a striatum transplant (HX-STR) animals have significantly fewer neurons than either normal animals or animals with transplants of spinal cord (HX-SC), cerebellum (HX-CB), or neocortex (HX-CTX). The values represented by these graphs are presented in tables 1 and 2.

Fig. 5. Cell soma size distribution in ipsilateral CN of adult operates: A. normal; B. hemisection alone; C. spinal cord transplant; D. cerebellum transplant; E. neocortex transplant; F. striatum transplant.

Fig. 6. Cell soma size distribution in ipsilateral CN of neonate operates: A. normal; B. hemisection alone; C. spinal cord transplant; D. cerebellum transplant; E. neocortex transplant; F. striatum transplant.

Fig. 7. Fluorescence photomicrographs of FG labeling in: A. normal CN; B. CN ipsilateral to hemisection alone; C. CN ipsilateral to hemisection with spinal cord transplant. Bar = 50 μm.

Fig. 8. Cell size distribution of Nissl stained and FG labeled neurons in CN. A. normal CN; most large neurons are FG labeled as are many small neurons. B. CN ipsilateral to hemisection alone. C. CN ipsilateral to hemisection with spinal cord transplant.

Fig. 9. Double staining of sections labeled with FG (B,D,F,G) and antibodies to GFAP (A), OX-42 (C), ED-1 (E), or MAP 1B (G) to identify cells in CN ipsilateral to a hemisection with spinal cord transplant. FG labeled cells (arrows) are GFAP, OX-42, and ED-1 negative. The DAB reaction product obscures FG labeling in the cytoplasm of MAP 1B positive neurons, but FG labeling remains visible within the nuclei. A, C, E, and G are brighfield photomicrographs. B, D, F, and G are fluorescence photomicrographs. Bar = 50 μm.

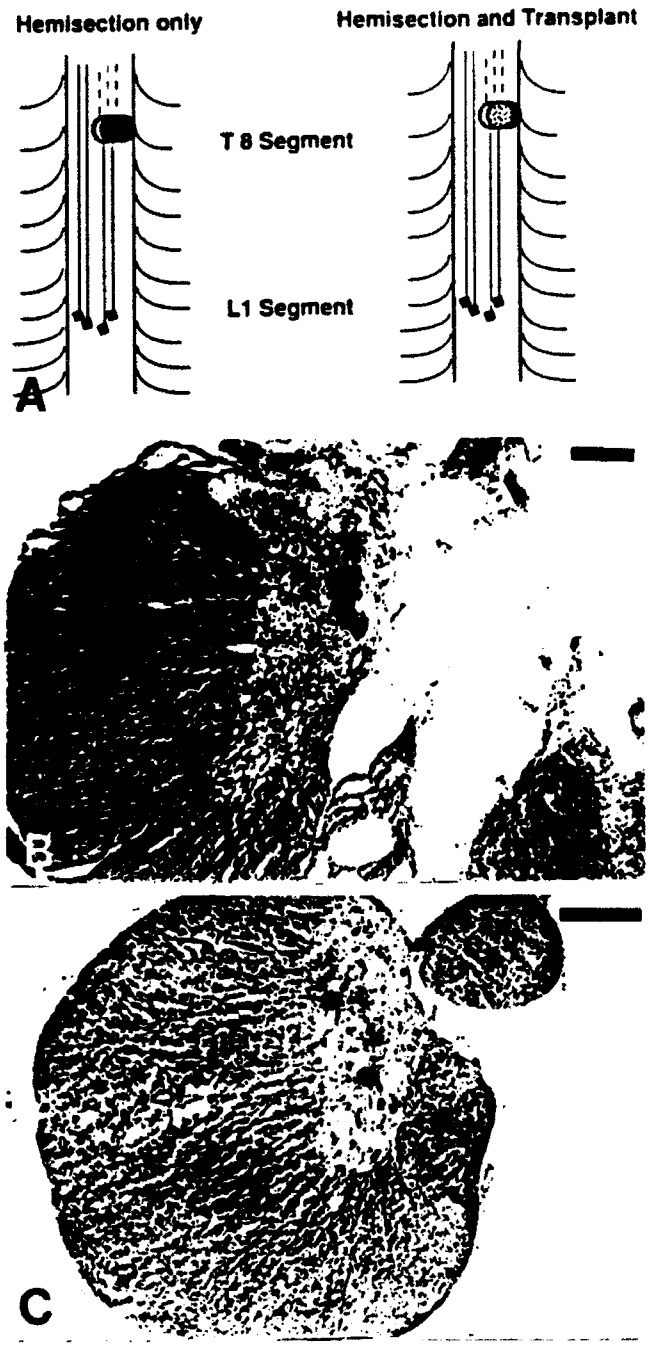

Hemisection only
Hemisection and Transplant
T 8 Segment
L1 Segment
A
B
C

Fig. 1

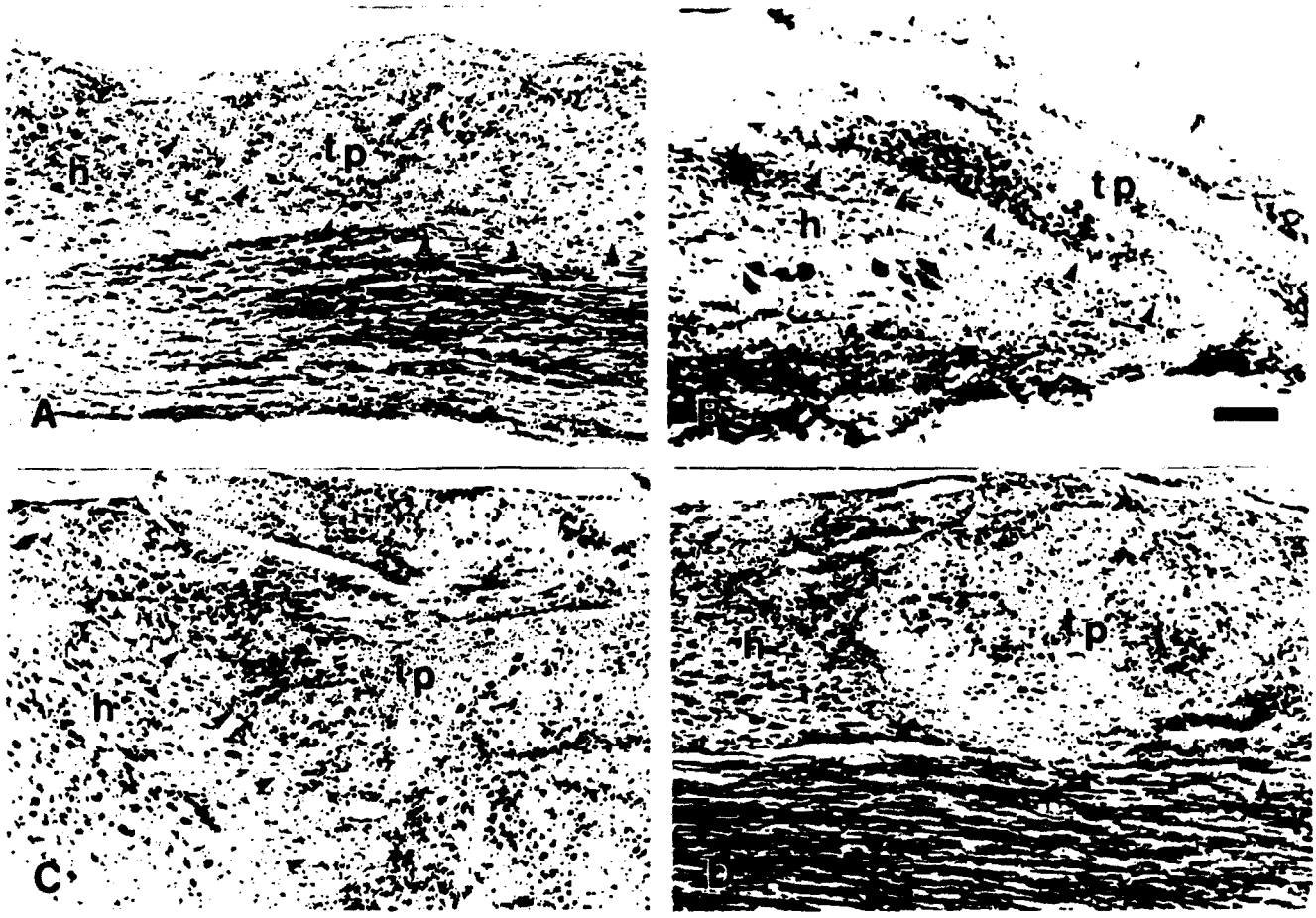


Fig. 2

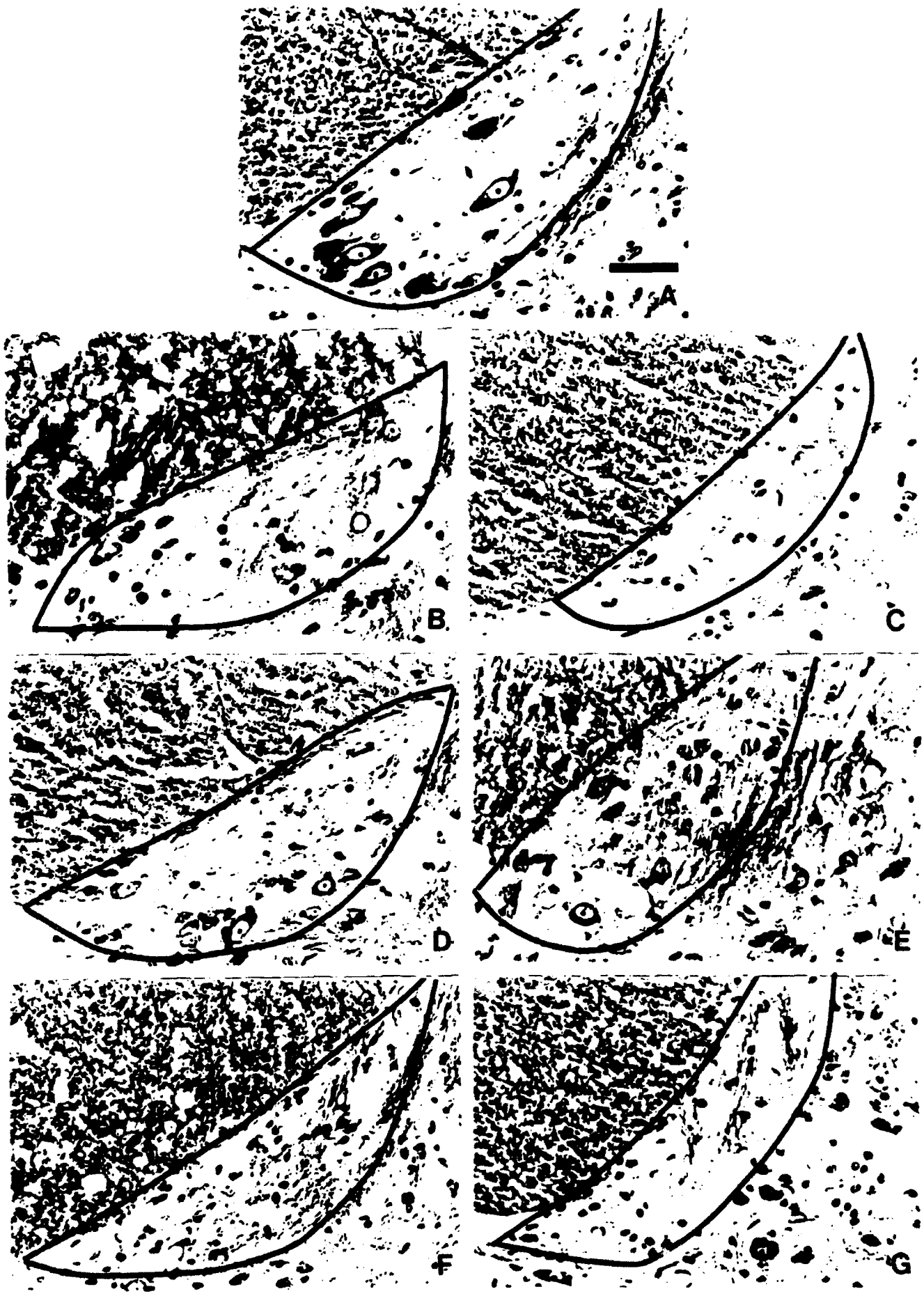
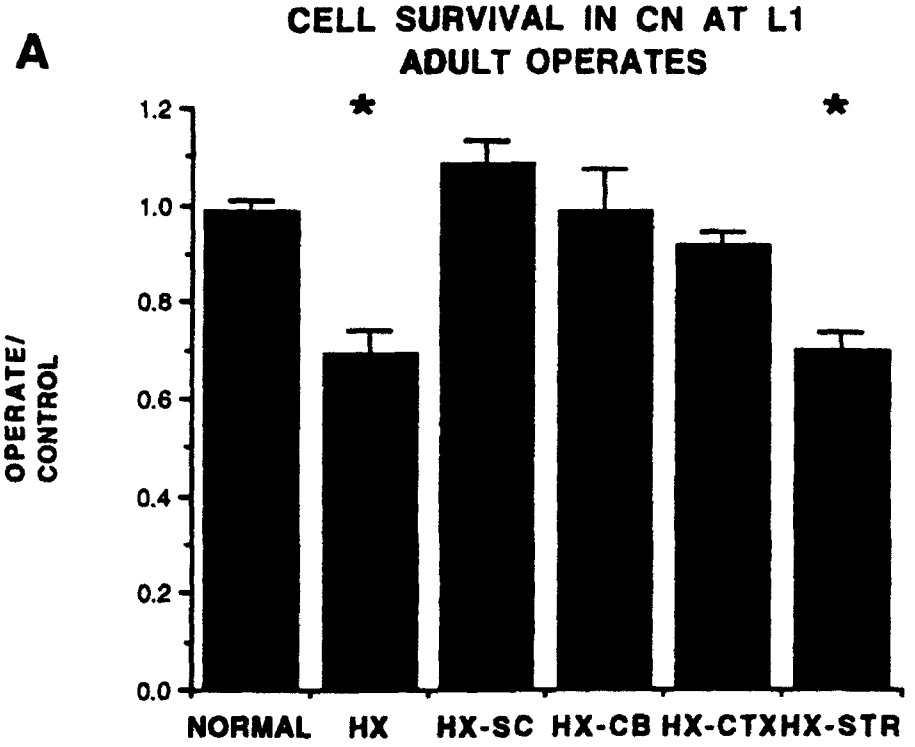


Fig. 3

A


CELL SURVIVAL IN CN AT L1
ADULT OPERATES
OPERATE/
CONTROL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
NORMAL
HX
HX-SC
HX-CB
HX-CTX
HX-STR

B

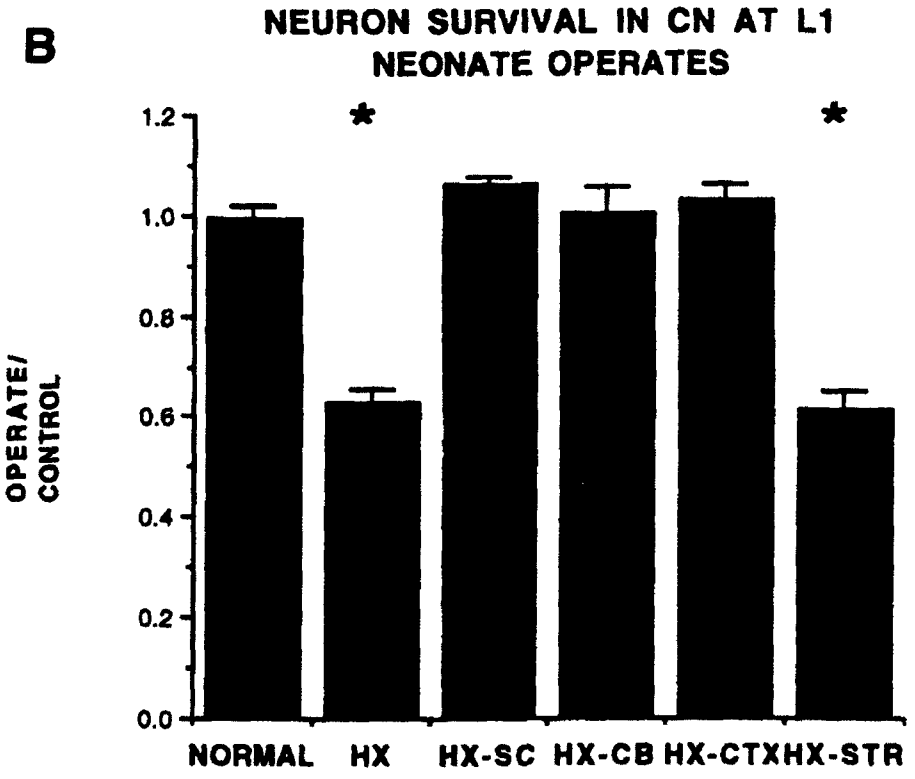
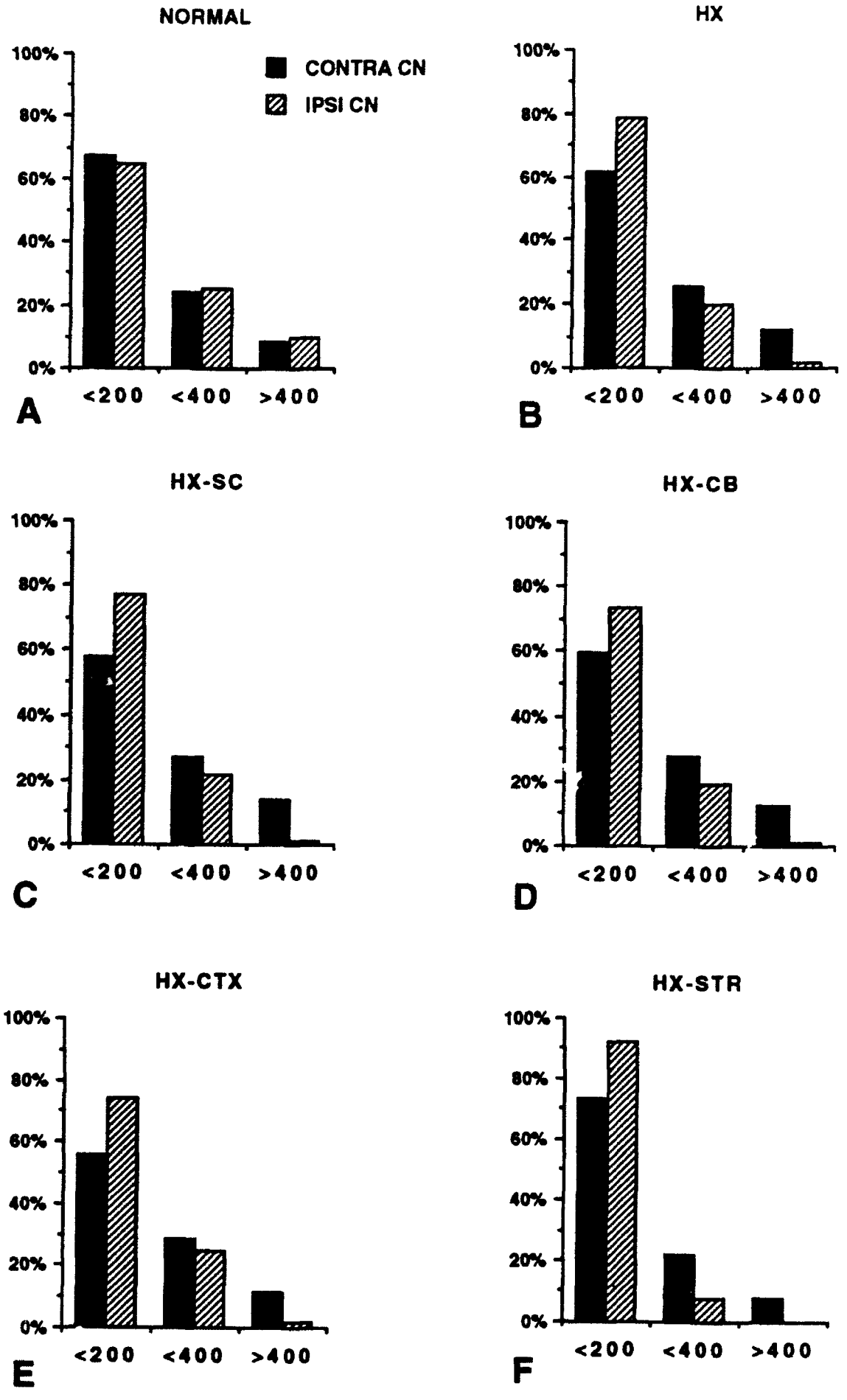

NEURON SURVIVAL IN CN AT L1
NEONATE OPERATES
OPERATE/
CONTROL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
NORMAL
HX
HX-SC
HX-CB
HX-CTX
HX-STR

Fig. 4


NORMAL
CONTRA CN
IPSI CN
100%
80%
60%
40%
20%
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<200
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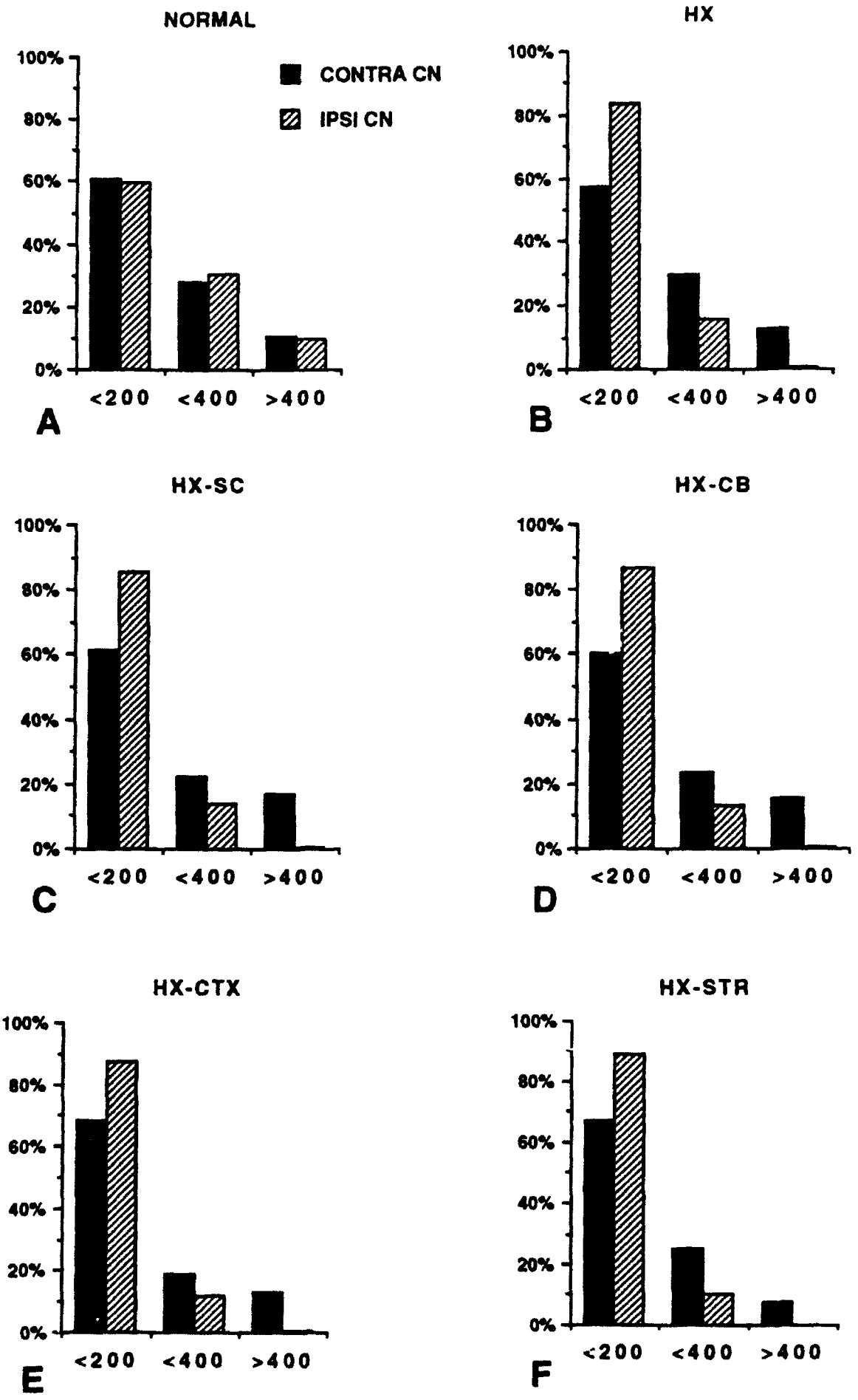
>400
A
HX
B
HX-SC
C
HX-CB
D
HX-CTX
E
HX-STR
F

Fig. 5


NORMAL
CONTRA CN
IPSI CN
100%
80%
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A
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C
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D
HX-CTX
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HX-STR
F

Fig. 6

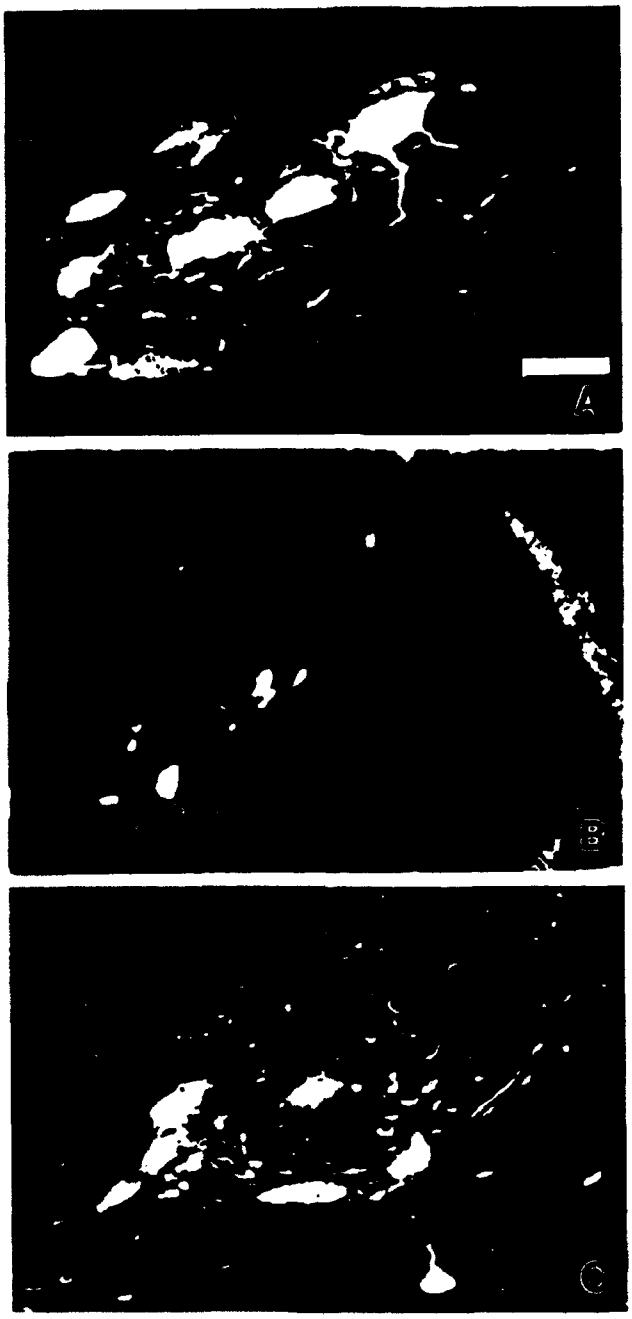
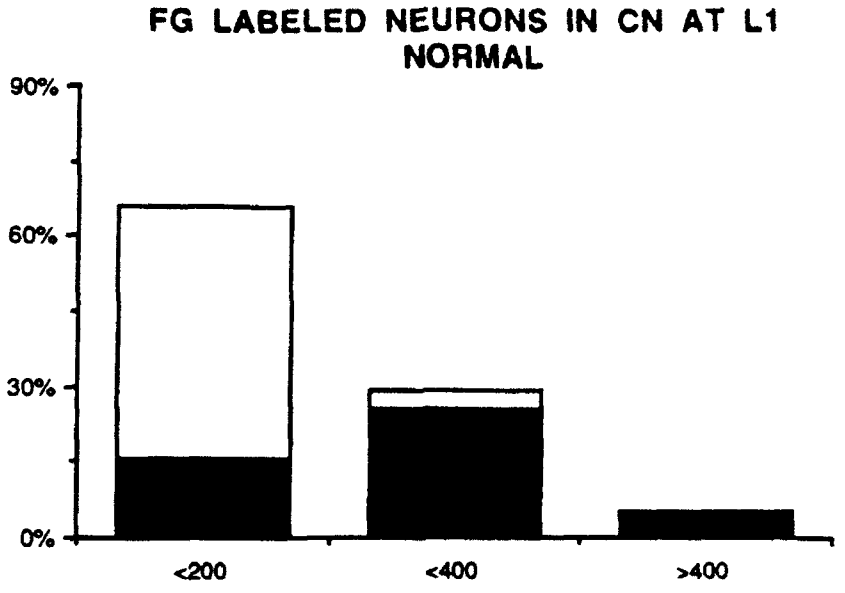


Fig. 7

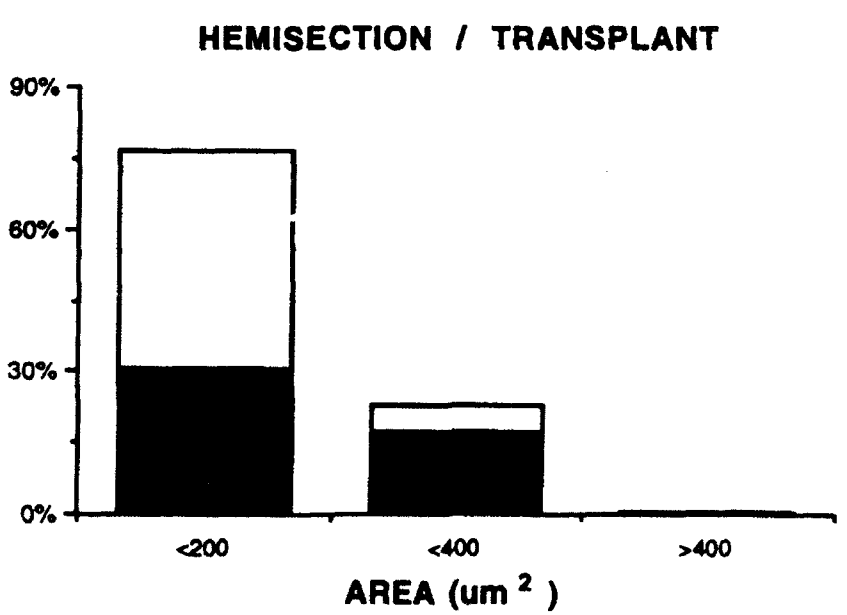
A


FG LABELED NEURONS IN CN AT L1
NORMAL
90%
60%
30%
0%
<200
<400
>400

B


HEMISECTION ONLY
90%
60%
30%
0%
<200
<400
>400
Unlabeled
FG Labeled

C


HEMISECTION / TRANSPLANT
90%
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<200
<400
>400
AREA (um 2)

Fig. 8

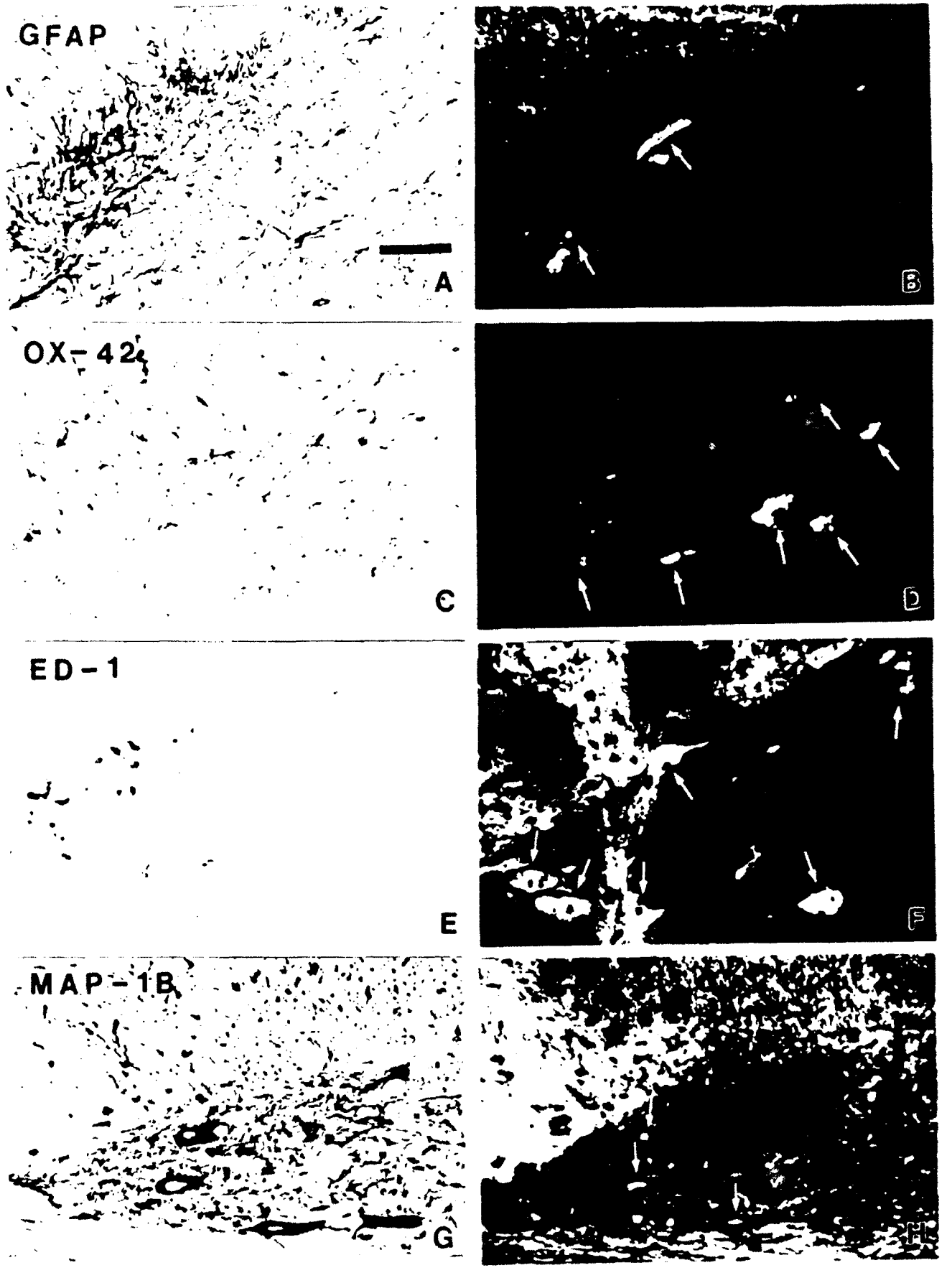

Fig. 9

Table 1. Size of Clarke's Nucleus, Mean Cell Size and Corrected Cell Number. Adult Operates, L1 spinal segment.

Group	Area of CN (μm^2)			Mean Cell Area (μm^2)			Cell Number		
	Left	Right	Ratio R/L	Left	Right	Ratio R/L	Left	Right	Ratio R/L
1 Control									
Mean (N=6) ± S.E.M.	47,794 ± 614	46,882 ± 958	0.981 ± 0.026	194.78 ± 5.15	203.74 ± 4.69	1.047 ± 0.014	18,58/ ± 1,147	18,289 ± 1,117	0.985 ± 0.024
2 Hemisection									
Mean (N=6) ± S.E.M.	43,471 ± 4,749	36,338* ± 3,920	0.832 ± 0.015	216.26 ± 15.74	153.91* ± 15.43	0.709 ± 0.040	17,011 ± 2,579	12,078* ± 2,251	0.695 ± 0.045
3 Hemisection/ E14 Spinal Cord									
Mean (N=6) ± S.E.M.	45,627* ± 2,995	40,207 ± 2,822	0.883 ± 0.037	226.31* ± 9.30	159.63 ± 7.13	0.708 ± 0.033	15,053 ± 2,687	16,098 ± 2,595	1.085 ± 0.050
4 Hemisection/ E15 Cerebellum									
Mean (N=5) ± S.E.M.	45,764* ± 4,448	40,905 ± 4,132	0.892 ± 0.010	220.78* ± 19.74	153.45 ± 7.43	0.707 ± 0.035	14,791 ± 2,154	14,191 ± 1,842	0.987 ± 0.085
5 Hemisection/ E14 Neocortex									
Mean (N=6) ± S.E.M.	50,103* ± 1,149	43,823 ± 1,325	0.875 ± 0.017	221.60* ± 11.02	168.42 ± 8.54	0.764 ± 0.031	16,812* ± 2,416	15,469 ± 2,491	0.914 ± 0.031
6 Hemisection/ E14 Striatum									
Mean (N=6) ± S.E.M.	40,119* ± 1,188	30,649 ± 1,093	0.764 ± 0.013	185.07* ± 7.01	121.24 ± 5.142	0.657 ± 0.025	20,823* ± 1,305	14,582 ± 1,332	0.699 0.0367
	1 > 2,3,4,5,6 3,4,5 > 6			1 > 2,3,4,5,6			1,3,4,5 > 2,6		

* - Right differs from left ($p < 0.05$) using paired comparison t-test. Overall significant difference among group ratios determined by Kruskal-Wallis one way ANOVA ($p < 0.05$). Individual post hoc comparisons made with the Wilcoxon-Mann-Whitney test corrected for multiple comparisons.

Table 2. Size of Clarke's Nucleus, Mean Cell Size, and Corrected Cell Number. Neonate Operates, L1 spinal segment.

Group	Area of CN (μm^2)			Mean Cell Area (μm^2)			Cell Number		
	Left	Right	Ratio R/L	Left	Right	Ratio R/L	Left	Right	Ratio R/L
1 Control									
Mean (N=6) ± S.E.M.	43,078 ± 3,791	44,592 ± 3,588	1.041 ± 0.023	212.22 ± 14.96	214.22 ± 16.64	1.008 ± 0.023	18,070 ± 1,348	17,953 ± 1,513	0.992 ± 0.031
2 Hemisection									
Mean (N=6) ± S.E.M.	44,055 ± 2,617	27,866* ± 1,911	0.632 ± 0.020	226.68 ± 18.69	142.77* ± 12.74	0.639 ± 0.026	15,974 ± 2,180	10,080* ± 1,423	0.631 ± 0.025
3 Hemisection/ E14 Spinal Cord									
Mean (N=6) ± S.E.M.	42,146* ± 2,211	33,613 ± 2,205	0.799 ± 0.041	234.80* ± 21.19	139.06 ± 2.94	0.620 ± 0.063	14,626 ± 2,216	15,596 ± 2,510	1.060 ± 0.020
4 Hemisection/ E15 Cerebellum									
Mean (N=6) ± S.E.M.	41,120* ± 968	31,447 ± 1,177	0.764 ± 0.014	231.17* ± 17.41	137.92 ± 6.30	0.606 ± 0.030	16,083 ± 1,216	16,109 ± 1,380	1.005 ± 0.054
5 Hemisection/ E14 Neocortex									
Mean (N=6) ± S.E.M.	44,758* ± 2,086	36,353 ± 2,108	0.811 ± 0.021	203.618* ± 10.02	127.82 ± 8.16	0.633 ± 0.044	17,391 ± 2,898	17,476 ± 2,363	1.029 ± 0.036
6 Hemisection/ E14 Striatum									
Mean (N=6) ± S.E.M.	37,531* ± 5,710	26,639 ± 4,946	0.689 ±0.046	174.28* ± 21.41	114.83 ± 13.67	0.669 0.039	18,617* ± 1,253	11,587 ± 1,295	0.616 ± 0.033
	1 > 2,3,4,5,6 3,4,5 > 2			1 > 2,3,4,5,6			1,3,4,5 > 2,6		

* - Right differs from left ($p < 0.05$) using paired comparison t-test. Overall significant difference among group ratios determined by Kruskal-Wallis one way ANOVA ($p < 0.05$). Individual post hoc comparisons made with the Wilcoxon-Mann-Whitney test corrected for multiple comparisons.

Table 3. Nissl Stained and Fluoro-gold Labeled Neurons in CN
Adult Operates, L1 Spinal Segment

Group	Nissl Stained Neurons			Fluoro-gold Labeled Neurons		
	Left	Right	Ratio R/L	Left	Right	Ratio R/L
1 Control						
Mean (N=4) ± S.E.M.	18,496 ± 1,159	18,399 ± 1,361	0.993 ± 0.024	6,277 ± 834	5,789 ± 913	0.912 ± 0.032
2 Hemisection						
Mean (N=4) ± S.E.M.	14,816 ± 3,220	9,450* ± 2,554	0.623 ± 0.041	6,159 ± 363	3,162* ± 453	0.515 ± 0.071
3 Hemisection/ E14 Spinal Cord Mean (N=4) ± S.E.M.	15,159 ± 2,986	15,397 ± 2,878	1.022 ± 0.012	6,929 ± 948	6,320 ± 530	0.942 ± 0.090
	1,3 > 2			1,3 > 2		

* - Right differs from left ($p < 0.05$) using paired comparison t-test. Overall significant difference among group ratios determined by Kruskal-Wallis one way ANOVA ($p < 0.05$). Individual post hoc comparisons made with the Wilcoxon-Mann-Whitney test corrected for multiple comparisons.

APPENDIX 8

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12

Sprouting and Regeneration in the Spinal Cord

Their Roles in Recovery of Function After Spinal Injury

Michael M. Goldberger, Marion Murray, and Alan Tessler

Department of Anatomy and Neurobiology, The Medical College of Pennsylvania, Philadelphia, Pennsylvania 19129

Injury to the brain or spinal cord is followed by a period of severe depression of function which is then succeeded by some recovery of function. The mechanisms underlying both the loss of function and the recovery of function are incompletely understood. After spinal transection in adult cats, there is complete loss of descending control of motor function owing to interruption of the descending motor pathways (1). This effect of the transection is permanent. There is also a depression of reflex function caudal to the lesion, the reasons for which are not yet evident. This reflex depression is not permanent; most reflexes show recovery and some may become hyperactive (2). Eventually, recovery of reflex locomotion, as elicited by a treadmill, can be demonstrated (3,4). The mechanisms underlying the remarkable recovery of reflex behavior remain unidentified although there are changes within the spinal cord caudal to the transection that may contribute to recovery and indeed are suspected of doing so (see ref. 5 for review). Thus a permanent loss of voluntary movement and a transient loss of reflex function are seen after the same lesion. The permanence of the loss of descending control is due to the failure of descending pathways to regenerate. The recovery of reflex behavior implies plasticity in spinal segments below the lesion that have been indirectly affected by the transection. Identification of the loci of the impairment and particularly of the nature of the recovery is useful as a guide to which systems should be examined further to reveal the nature of that plasticity that leads to recovery of function. One naturally occurring anatomical mechanism that can account for recovery of function is collateral sprouting. New studies using fetal transplants into injured spinal cord offer the possibility of enhancing this naturally occurring plasticity and achieving greater recovery of function.

PLASTICITY IN THE INJURED SPINAL CORD

Collateral Sprouting

Definition and Demonstration

Collateral sprouting (reactive reinnervation) refers to an expansion of the terminal

field of undamaged axons in response to partial denervation of target neurons by removal of converging systems. In adults, the amount of growth is generally limited and is demonstrable only within the normal terminal field as an increase in density of the projection. The absence of extension of a projection beyond its normal limits in the adult has been frequently confirmed, although it has sometimes been interpreted as indicating an absence rather than a limitation on sprouting (6). Sprouting appears to be a regulated rather than a random occurrence so that certain systems are more likely to increase their projections in response to a specific lesion than others. There are also conditions under which sprouting of a specific pathway does not occur subsequent to a lesion that appears to denervate its target (7–11). In at least some of these cases, these results suggest a target regulation of sprouting.

If lesions are made in developing animals before the adult patterns of axonal projections and synaptic contacts are established, the sprouting response may include extension of the projection to regions normally not supplied or only poorly supplied by the undamaged system. Another form of plasticity, compensatory sprouting or pruning, refers to the proliferation of collaterals located proximal to the site of axotomy (12). To elicit this form of sprouting, the neuron must be damaged. This form of plasticity, which sometimes results in aberrant projections, has been shown most clearly in developing neurons.

The controversies that have arisen over the truth and consequences of sprouting in spinal cord are in part due to unappreciated methodological difficulties in demonstrating differences in densities of projections. Detection of an increased density in a normal projection requires quantitative methods sufficiently sensitive to reveal a difference between the experimental and control projections. In most cases, sprouting has been examined using light microscopic tract tracing methods, and the contralateral projection is frequently used as the control. Intra-animal comparisons of density of the control and sprouted projections may be the most sensitive way to determine the extent of modification of an afferent projection, but this requires establishing that the ipsilateral and contralateral projections are normally comparable in size and that the projections are uncrossed: if either of these requirements is unfulfilled, the appropriate corrections must be made. If axonal transport methods are used to identify the projection, e.g., HRP labeling, then comparable supply of the label to the control and the sprouting projections must be demonstrated. These methods used with interanimal comparisons are too insensitive to recognize differences in density if there is considerable normal interanimal variability in the density of projections. The interpretation of the results will be additionally confounded by the difficulty of demonstrating comparable delivery of the label to the test and experimental projections in different animals. Immunocytochemical staining of specific pathways is another light microscopic method that has been used to demonstrate sprouting. Differences in the apparent density of projections can, however, be attributable to increased synthesis or transport of the labeled substance. No control for this possibility has been devised as yet.

Quantitative electron microscopy has also been used to study sprouting (reactive reinnervation). Changes in the synaptic complement in terminal fields of target nuclei with recovery of terminal number toward normal levels can be shown after lesions. These changes indicate proliferation of terminals by undamaged systems leading to reinnervation of the denervated targets. The major procedural problem in these ultrastructural studies is dealing with the limited sample size and the requirement for methods to assess the extent of shrinkage and other changes in neuropil.

It is considerably easier to demonstrate sprouting of axonal projections in neonatal

animals. In addition to the increased projection density found in normal targets, the axons may also follow an aberrant course and form projections to inappropriate targets. Qualitative methods may therefore be sufficient to demonstrate aberrant projections. On the other hand, changes in the target (atrophy or cell death) are more likely to occur in a developing system following a lesion and compensation for these changes is required in order to interpret the results.

It should be noted that there are alternative interpretations of the increased density of projections seen after partial denervation. For example, projections normally not revealed, perhaps because the axons and terminals are below light microscopic resolution or because they contain inadequate amounts of label or immunoreactive material, may become demonstrable only under conditions of increased activity, without an actual increase in the number of contacts that are made. Such a possibility cannot be easily ruled out although there is relatively little evidence in support of this interpretation (45) and it is not supported by ultrastructural data.

Evidence for Plasticity Induced by Lesions in the Adult Spinal Cord

A considerable body of evidence has accumulated both supporting and questioning collateral sprouting after lesions in the adult and neonatal spinal cord since the first report in 1958 (13). The source of many of the controversies now appears to be accounted for by the methodological difficulties associated with demonstrating sprouting in the adult spinal cord.

Sprouting of Dorsal Root Axons

The Spared Root Preparation. This preparation is the classic model for demonstrating dorsal root sprouting (8,13–16). Several dorsal roots are cut rostral and caudal to the test dorsal root. After survival times adequate to permit sprouting by the central processes of the spared root and disappearance of the degeneration products from the cut roots, test lesions are made of the spared root and the contralateral control root. Using a newly developed method for identifying degenerating axons, Liu and Chambers (13) described an increased density of projection by the spared root axons on the experimental side, which they interpreted as sprouting of the spared root. They also described an extension of the projection of lumbar dorsal root axons to levels of Clarke's nucleus several segments rostral to the level at which control axons terminated, an increased projection which would be considered an aberrant projection. Their results were largely confirmed by Goldberger and Murray (8), with the exception of the aberrant extended projection. This disparity in results can probably be accounted for by methodological problems associated with the suppression step used in this procedure. These results have been criticized recently by Kruger and his colleagues (17–19) who attributed the apparent sprouting to inadequate controls. Rodin et al. (17) attempted to replicate the Liu and Chambers model but used transganglionic labeling with HRP instead of degeneration stains to compare the projections of test and experimental roots. They were unable to identify a difference in density of projection between a spared root in an experimental animal and the comparable root in another unoperated animal and therefore concluded that sprouting did not occur. Since the spared root projection was determined in one series of animals, and compared to the projection of a root at the same level in another group of animals, these authors were unable to make appropriate compensations for the very large differences between animals in numbers of dorsal root ganglion (DRG) cells and therefore numbers of centrally projecting axons at the lumbar levels. Since the number of DRG cells may vary as much as 50% from one animal to another (20), the sensitivity of their method was too

low to support their conclusions. Polistina et al. (21) took advantage of the fact that the number of DRG cells in a lumbar ganglion does not vary greatly between the two sides of the same animal (20,22,23) and therefore used intra-animal comparisons supported by an internal control (counting the number of labeled neurons) to demonstrate comparable labeling of the test and control projections. Their results showed increased densities of the spared root projections that were statistically significant but no evidence of extension of projections to aberrant targets. They also showed that the normal interanimal variability is sufficient to mask these differences when uncontrolled interanimal comparisons are used.

Histochemical and immunocytochemical methods were used by McNeill and colleagues (15,16) to examine the density of projections of the spared T9 root in rat. Fluoride-resistant acid phosphatase (FRAP), a marker for axons originating from a class of small DRG neurons, was shown to increase in density in the superficial layers of the dorsal horn on the spared root side compared to the control side while calcitonin gene-related peptide (CGRP), a marker for terminals originating from other classes of dorsal root ganglion neurons, was shown to label approximately twice as many terminals in the dorsal horn at levels near the spared root compared to the control side. Further ultrastructural evidence supporting plasticity in dorsal root terminals of the spared root is provided by Zhang et al. (unpublished results), who have shown increased numbers of terminal contacts and increased lengths of postsynaptic densities associated with complex terminals arising from small DRG neurons on the spared root side. Thus the immunocytochemical studies of specific populations of dorsal root axons support the results from controlled horseradish peroxidase (HRP) and degeneration methods, all indicating increased densities of dorsal root projections in the spared root preparation. The electron microscopic (EM) studies further suggest that the sprouting is associated with terminal proliferation.

A variation of the spared root preparation has been used in two studies in which neurotoxic compounds, injected into the peripheral fields of spinal nerves, are transported to the DRG. A proportion of DRG cells are killed, but those spared can be examined for evidence of sprouting. Pubols and Bowen (24) injected ricin, a compound that kills a variable number of DRG cells, into sciatic nerves 2 weeks to 2 months before sacrifice. Six days prior to sacrifice they injected ricin into the contralateral (control) sciatic nerve. HRP injections into dorsal rootlets were then used to compare labeling on the two sides. These authors found considerable variability in labeling of the projections between the two sides, which appeared to be a function of variations in the amounts of HRP injected, but failed to find consistent differences between control and experimental sides. They interpreted this finding as absence of evidence for sprouting. The crucial controls in these experiments would have been first to demonstrate loss of axons after ricin injection on the two sides and then to compare projections in animals in which they could demonstrate comparable HRP uptake by dorsal roots of the two sides. Their interpretation is further complicated by the fact that sprouting is likely to have occurred on the control side at the 6 day postinjection survival time used (25) so that comparable labeling of the two sides might have been expected under well-controlled conditions. Lamotte et al. (26), using a similar paradigm, injected pronase, a combination of proteolytic enzymes, into the sciatic nerve. Pronase is also transported to the DRG where it kills ganglion cells. They later injected HRP and wheat germ agglutinin (WGA)-HRP into the cut saphenous nerves and measured the area in dorsal horn supplied by labeled axons. Controls showed evidence for comparable and symmetrical labeling in normal animals using their HRP methods, and demonstrate loss of unmyelinated axons in the dorsal

roots caused by the pronase treatment. These workers found an increase in area labeled with HRP and an increase in the number of labeled terminals on the experimental side which they interpreted as evidence for sprouting by central processes of saphenous axons in response to loss of central processes of the sciatic axons. Whether the sprouting from the saphenous nerve extended into novel territories or simply increased within its normal territory is difficult to resolve given the unknown extent of overlap of the two projections in the dorsal horn (27,28,29a).

Lesions within the CNS that partially denervate targets of dorsal root axons can also elicit sprouting of dorsal roots. This was first reported using hemisection in the cat (29) and more recently was confirmed with immunocytochemical methods (30), although the same lesion in rats elicited clear evidence of dorsal root sprouting only in young animals (31a). Rodin and Kruger (18) again used transganglionic transport of HRP to look for differences in dorsal root projections ipsilateral and contralateral to a thoracic hemisection. They reached the conclusion that sprouting does not occur because they could find no consistent differences between projections of experimental or control roots; as in their previous study, they used interanimal comparisons and did not control for differences in amount of HRP transported, an approach that is not likely to be sensitive to differences in the projections between animals or technical differences in delivery of the label. More recently anterolateral cordotomy in the monkey has been shown to produce rearrangement of primary afferents within but not outside of regions of normal termination, a change consistent with denervation-induced sprouting (31).

Peripheral Nerve Lesions. It is well known that peripheral nerve lesions are often followed by regeneration of the cut axons. It has more recently been shown that these lesions also cause death of some DRG neurons (20) and degeneration of their central processes (32). These lesions also evoke a variety of changes in the surviving ganglion cells that appear to be reversed after regeneration of the peripheral process occurs (33). These changes include decreased synthesis of some peptide transmitters (34–36); up-regulation of synthesis of other peptides (37,38); increased synthesis of growth-associated proteins (39) and cytoskeletal proteins (40); and changes in morphology of the central terminals (41). Resection of the sciatic nerve, which prevents regeneration and partially denervates central targets, combined with crush of saphenous nerve, which stimulates saphenous regeneration, elicited central sprouting by the saphenous dorsal root afferents, which appeared to exceed the normal central projection of the saphenous nerve (42). These studies suggest that the changes in DRG neurons associated with the regenerative responses to peripheral nerve lesion can enhance the growth responses of the axons within the CNS (43). McMahon and Kett-White (44) tested this hypothesis more directly in adults by cutting the peripheral nerve of the spared root. This combined lesion enhanced sprouting by axons in the spared root and also increased the area supplied by the spared root. This important observation indicates that the usual spatial limitation of sprouting in the adult can be overridden by an additional manipulation that increases the growth potential of the adult axon.

Of particular interest in considering plasticity associated with peripheral lesions is the evidence that very marked and long lasting modifications of central pathways within the spinal cord (45,46) and extending to changes in cortical representations (47–49) occur after peripheral nerve lesions.

Sprouting of Central Pathways

Central pathways have also been shown to sprout. Most of the evidence in favor of

central sprouting in the adult spinal cord comes from studies in which the cord has been deafferented by dorsal rhizotomies. Tessler et al. (50–52) first used immunocytochemistry and radioimmunoassay to demonstrate, in the cat, that deafferentation, which eliminates the major SP input to laminae I and II of the dorsal horn, elicits a recovery of SP, but not somatostatin. They interpreted these data as indicating sprouting by intraspinal SP-containing neurons. These observations were subsequently confirmed in the cat (19) and rat (11). The population of synaptic terminals in lamina II also changes following deafferentation, with a loss of all of the characteristic scalloped terminals and some of the simple terminals, but with no loss in total number of terminals (25). This observation suggests that complete deafferentation evoked rapid replacement of lost dorsal root terminals by simple terminals from intrinsic sources. Recent quantitative analyses by Zhang et al. have shown that the number of simple SP-labeled terminals increases after deafferentation, a finding consistent with the interpretation of reactive reinnervation (Fig. 1). Beattie et al. (10) compared synaptic input after transection to two nuclei in sacral spinal cord, Onuf's nucleus, and the sacral

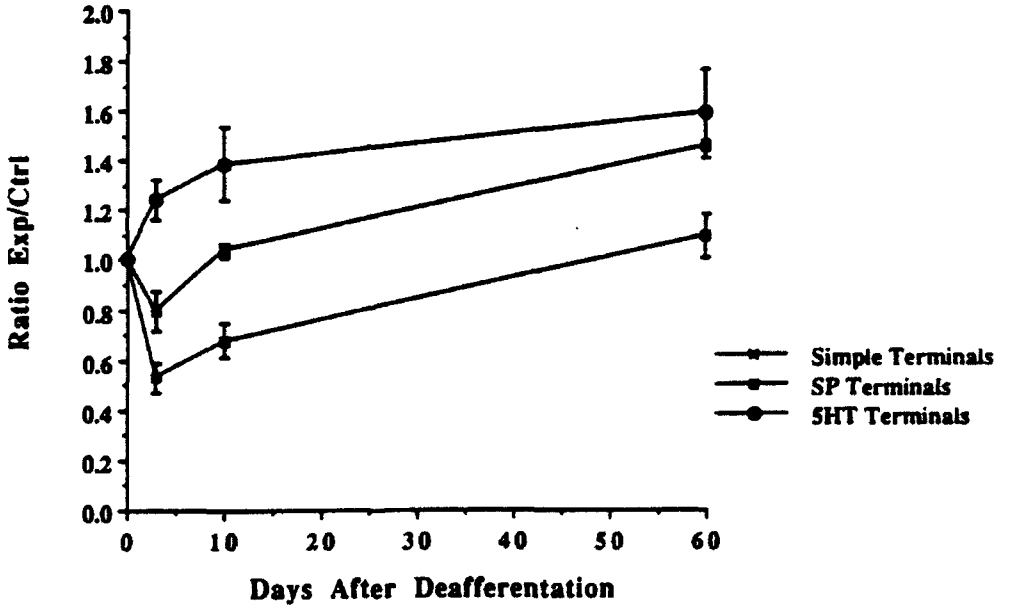

FIG. 1. Changes in the total number of simple terminals, number of simple terminals immunoreactive for SP, and number of simple terminals immunoreactive for 5HT in lamina II after unilateral lumbosacral deafferentation in the rat. The results are expressed as a ratio of counts on the control lamina II to counts in the deafferented lamina II. Postembedding immunocytochemical methods were used to identify SP and 5HT terminals. Note that the number of simple terminals decreased postoperatively and then increased to above normal levels, indicating proliferation in response to deafferentation. Immunocytochemical analyses indicated that part of the increase can be attributed to increased number of SP-containing terminals, which show a time course of change similar to that for total simple terminals, and part can be attributed to 5HT-containing terminals, which show a different time course. The time course of change of number of terminals parallels the time course of change of density of immunocytochemical reaction, shown light microscopically (11).

parasympathetic nuclei. They found evidence for synaptic replacement in Onuf's nucleus but permanent denervation in the sacral parasympathetic nucleus, suggesting that sprouting may be regulated differently in these two nuclei. Different results were reported by Chung (53), however, who counted synaptic thickenings in the sacral dorsal horn after unilateral deafferentation in the rat. They found a decrease in numbers of contacts compared to control animals on both operated and unoperated sides during the first postoperative week.

Serotonergic pathways that descend from the brainstem also sprout in response to deafferentation. Wang (11), using a complete lumbosacral deafferentation model, showed a twofold increase in density of 5-hydroxytryptamine (5HT) immunoreactivity in lamina I and II. Polistina (21) showed in a spared root preparation that the density of the 5HT projections increases at denervated spinal levels rostral to the zone where the spared root axons have increased their projections. These studies indicate that the descending serotonergic pathway will increase its projection density in response to removal of convergent dorsal root projections to the dorsal horn. EM-immunocytochemical studies by Zhang et al. indicate that serotonergic-containing terminals also increase in lamina II after deafferentation (Fig. 1). Interestingly, in the same preparation, the descending noradrenergic projection to the dorsal horn showed no change in density indicating a selectivity in which systems sprout in response to deafferentation (11).

Finally, hemisection lesions have also been shown to modify the projections of corticospinal neurons in the adult monkey (54). In this study HRP was injected into the spinal cord contralateral to a hemisection and increased numbers of labeled neurons were found in the cortex ipsilateral to the lesion, suggesting sprouting of projections across the midline or by ipsilaterally projecting corticospinal axons.

Evidence for Plasticity Induced by Lesions in Neonatal Animals

Lesions in neonatal animals often result in greater sprouting than in adults and as a result the issue of neonatal sprouting seems less controversial. This increased plasticity may account for the greater recovery of function associated with lesions in developing animals. Usually the extent of plasticity which can be evoked by lesions made in the neonate declines toward adult levels during the postnatal period.

Sprouting of Dorsal Root Axons

The classic spared root preparation has not been studied in neonatal animals. A similar paradigm is however provided by experiments in which capsaicin is injected (55,56). Capsaicin, when injected in neonatal rat, destroys most of the small DRG cells, eliminating about 90% of the unmyelinated axonal input to the dorsal horn. The spared large DRG cells, which normally project to deeper layers of the dorsal horn, develop aberrant projections into the denervated superficial layers as well and form terminals there.

Hulsebosch and colleagues (57,58) have shown that administration of antibodies to NGF to otherwise normal neonatal rats had the unexpected effect of eliciting sprouting of unmyelinated axons in the dorsal root and in Lissauer's tract. Administration of anti-NGF antibody results in the death of some DRG cells (59) with the possibility that small DRG cells are more vulnerable than the large cells. The surviving DRG cells appear to emit greater numbers of axonal collaterals in response to the loss of other DRG cells, and this form of sprouting was clearly demonstrable by axon counts both in the dorsal root and intraspinally (57,58). The mechanism by which this sprouting is elicited is likely to be neutralization of the nerve growth factor (NGF)

necessary for the survival of small DRG cells and which is normally supplied to them by axonal transport from the periphery. The loss of this class of dorsal root ganglion cells thus elicits a response similar to that achieved by capsaicin delivered neonatally. As in the capsaisin studies, the surviving large DRG cells appear to project into aberrant regions of the spinal cord, i.e., Lissauer's tract.

The effects of peripheral nerve lesions in neonates have been compared with the same lesion in adults. Peripheral nerve section in neonates, in contrast to adults, produces massive DRG cell death, resulting in a much greater denervation of the dorsal horn. The neonatal lesion therefore provides a greater stimulus for sprouting by the spared ganglion cells than the adult lesion (20,59). Peripheral nerve section leads to loss of FRAP staining in the part of the dorsal horn supplied by the injured ganglion cells. In neonates, but not in adults, the area of depletion in the dorsal horn is rapidly filled in by the sprouting of FRAP-containing afferents from adjacent intact ganglion cells (60). Similarly, neonatal sciatic nerve lesions elicit an expansion in the saphenous terminal field in the dorsal horn. This sprouting is less extensive in animals more than 5 days old, indicating the postnatal critical period for this form of plasticity (61). Fitzgerald et al. (62) used physiological methods supported by HRP labeling to demonstrate sprouting of specific types of dorsal root afferents into inappropriate central fields as a result of neonatal sciatic nerve lesion. The terminals formed by the aberrant projections were appropriate to the new target areas, suggesting target regulation of terminal morphology.

Sprouting of Central Pathways

Neonatal deafferentation by rhizotomy (63) or by administration of capsaisin (64) has been used to demonstrate sprouting by serotonergic axons in the dorsal horn. Capsaicin injections on the day of birth, eliminating most of the central projections of the small DRG cells, elicited sprouting by descending 5HT axons into aberrant regions of the dorsal horn. Dorsal rhizotomy on postnatal day 5 evoked increased 5HT densities comparable to the twofold increase shown after deafferentation in adults (11). However, the sprouting in neonates extends into deeper layers of the dorsal horn than in the adult. These studies taken together suggest a critical period within the first 5 days postnatal that limits the extent of aberrant sprouting of the serotonergic fiber systems in response to rhizotomy.

Pyramidal lesions interrupt the corticospinal pathway and evoke sprouting of corticospinal axons to the contralateral spinal cord (65). Cerebral hemispherectomy also evokes sprouting to spinal areas (66). Both studies report greater effects when the lesions are made in the neonate than in the adult.

In summary, there is convincing anatomical evidence for sprouting in the spinal cord. Dorsal roots have been shown to increase their terminal density in adult spinal cord in a variety of experimental paradigms. In most of the experiments in which sprouting could not be demonstrated, methods were used that did not take into account normal variability either between animals or in the methods used to test sprouting. Similarly there is generally consistent evidence of sprouting by central systems in response to partial deafferentation. Most quantitative studies at both EM and light microscopic levels indicate that sprouting is associated with marked, not trivial, increases in projection densities, which may be sufficient to restore numbers of terminals on partially denervated targets to close to normal levels. Expansion of terminal fields into novel territories in adults has been demonstrated most convincingly in cases where additional manipulations are made, e.g., addition of peripheral nerve injury to the lesion paradigm. This suggests that the limitation on sprouting in adults

can be overridden by increasing the metabolic drive of the sprouting neurons. In general, greater sprouting is seen when lesions are made before development has ceased, and this neonatal sprouting may include expansion into areas not normally supplied without additional manipulations. Naturally occurring plasticity in adults as well as in neonates represents a major compensatory response to CNS lesions, which needs to be taken into account in considering the consequences of CNS lesions, the extent of recovery of function, and ways of improving the extent of recovery of function.

Functional Recovery

Each of the three classical preparations that has been used to study sprouting in the spinal cord, i.e., hemisection, spared root, and deafferentation, has also been studied behaviorally to determine whether recovery of function can be observed. Not only is recovery of motor behavior documented but, in each case, the pathways that show sprouting have also been shown to have major roles in mediating recovery.

Spinal Cord Hemisection

Hemisections were made in the lower thoracic cord of the cat, sparing the dorsal columns so that motor impairments resulting from loss of the major ascending pathway would not be confused with those associated with loss of descending input (30). The animals had been preoperatively trained to walk on a 30 cm wide "simple" runway as well as on more challenging runways, including a horizontal ladder, a grid, and a narrow runway; the time to cross and number of errors were recorded and a kinematic analysis of joint angle excursions carried out. The threshold for placing, the force of the positive supporting reaction, and the kinematics of placing and hopping reactions were measured. Recovery of basic locomotion could therefore be compared with recovery of locomotion requiring accurate placement of the hindlimb. Careful analysis shows a predictable pattern of recovery of function. For a brief period, the hindlimb on the hemisected side is severely paralyzed both for locomotion and for postural reflexes but by the end of the first week considerable recovery occurs. During the first week, basic locomotion on the wide runway and also reflex locomotion on a treadmill recover. Measurement of joint angle excursions during the step cycle, however, reveals a kinematic pattern that is clearly abnormal; furthermore, the animals frequently walk on the dorsum of the toes on the hemisected side. Postural reflexes also show an early stage of recovery. Although contact (hair-bend) placing never recovers, proprioceptive placing (placing in response to bending of some part of the limb) does recover. The amount of bending required to elicit placing, i.e., the threshold, is considerable and the response is clearly hypermetric. Similar effects are seen when the monopedal hopping response is elicited; the amount of passive displacement on the treadmill is exaggerated compared to normal and the response is hypermetric. The positive supporting response is also deficient compared to the intact side.

During the second postoperative week, another phase of recovery begins. The thresholds for placing and hopping decrease although not to normal values (Fig. 2). Hypermetria is also reduced. The force of the positive supporting response recovers to normal. Most dramatic, however, is the recovery of accurate limb placement during locomotion, which begins to occur at this time. Initially, many errors in foot placement are observed and this slows the animal's crossing of the runway. In time, the number of errors decreases and speed increases, but normal values are not attained. During this period, the kinematic pattern returns almost to normal and the animals stop walking on the dorsum of the foot.

Therefore the first phase of recovery con-

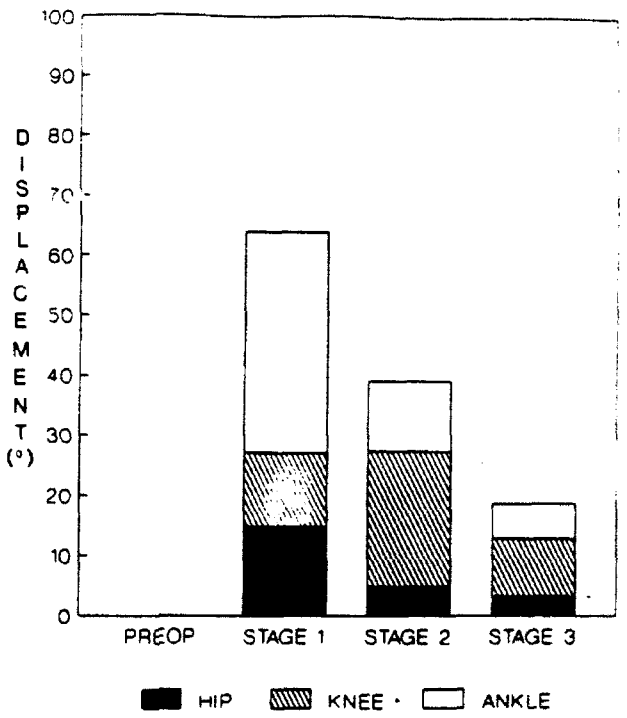

FIG. 2. This figure shows the amount of limb displacement required to elicit a placing response after spinal cord hemisection. The height of each bar corresponds to the total limb displacement, and the individual compartments correspond to the displacement of the hip, knee, and ankle. Preoperatively no displacement is required since the animals respond to hair stimulation. Immediately postoperatively, the amount of displacement is very large, especially at the ankle, but partial recovery takes place and becomes stable *(STAGE 3)*.

sists of recovery of basic locomotion and of crudely performed, high threshold postural reflexes. The second phase consists of refinement of the postural reflexes including lowering of their thresholds. The change in threshold may be due to a compensatory increase in afferent control of movements when descending control has been interrupted. Collateral sprouting of dorsal root afferents has been demonstrated after hemisection of the cat spinal cord by degeneration methods (29,30) and by immunocytochemistry (30). This sprouting of dorsal root terminals may represent a mechanism underlying the increase in reflex control.

Physiological changes have been observed after hemisection that are consistent with the notion of a compensatory increase in afferent control after a loss of descending input. Hultborn and Malmsten (67,68) demonstrated an increase in the strength of the monosynaptic stretch reflex and also in the cutaneous flexor reflex by using ventral root response to these different inputs. More recently, Pubols and her colleagues (69–71) have shown that the number of spinal cord neurons responding to cutaneous (sural nerve) inputs increases substantially after damage to descending pathways on one side of the cat spinal cord. Interestingly, the magnitude of the increase is similar to the number of neurons that possess subliminal sural nerve inputs in normal cats (72). These neurons produce EPSPs but not impulses upon sural nerve stimulation suggesting that the increase after the lesion is due not to the formation of new aberrant connections but rather to the strengthening

of already existing but weak connections. The strengthening of these connections could be mediated by sprouting of dorsal root terminals in the regions denervated by the lesion, denervation supersensitivity of neurons in such regions, or other unidentified mechanisms.

Spared Root Preparation

Since the spared root preparation was the first to be used to study sprouting (13), it is important to determine whether or not recovery of function can be demonstrated in this model. We have studied a group of cats with unilateral deafferentation, sparing the L6 root (73). Initially, the effect of the spared root deafferentation is quite marked. Locomotion is impaired, the partially deafferented limb is dragged, contact placing and the monopedal hopping reaction are abolished, and the positive supporting reaction is considerably weakened. These severe impairments are transient, however, and recovery begins during the first postoperative week. Basic locomotion, performed on a wide runway or a treadmill, returns, although the kinematic pattern used for locomotion is abnormal. Proprioceptive placing can be elicited, high threshold monopedal hopping responses return, and weight support begins to recover. There is thus an early recovery of basic locomotion and crudely executed postural reflexes. During the second week, a recovery of accurate limb placement on the complex runways is observed. Initially the animals make considerable numbers of misplacements but the errors decrease with time and the speed of crossing increases. There is continued recovery of the postural reflexes; contact placing returns, the frequency of placing to the lowest stimulus increases, the threshold for monopedal hopping decreases, and weight support increases. A normal kinematic pattern during locomotion is reestablished.

Thus the pattern of recovery is similar after hemisection and after spared root deafferentation: a lowering of the threshold for postural reflexes is associated with recovery of accurate limb placement and the return of a normal kinematic pattern used in locomotion. It is reasonable to hypothesize in both preparations that dorsal root sprouting increases the afferent control of movement and that this is responsible for the recovery of motor behavior. In both cases, there are alternative possibilities due to sparing of some of the descending pathways. After hemisection, the contralateral descending pathways are spared, and in spared root preparations the ipsilateral (as well as contralateral) descending pathways are available to mediate recovery. This possibility was tested by making contralateral hemisections in the hemisected animals and ipsilateral hemisections in the spared root animals after recovery had taken place. In both cases, most of the recovered behavior remained after the second lesion suggesting that it was the primary afferent control that was largely responsible for the recovery. This suggests that there may be a competitive interaction among the spared pathways after a lesion and they do not contribute equally to the recovery process.

There are physiological changes after partial spinal cord deafferentation which are consistent with the idea of increased afferent control mediated by the spared root in response to partial denervation of its terminal field. Pubols and Goldberger (74) found that there was a loss of responsiveness in the lateral dorsal horn acutely after spared root deafferentation sparing L6. There was then a recovery of responsiveness but the receptive field organization had changed in this portion of the dorsal horn. A permanent loss of proximal receptive fields was found, and this loss was compensated for by an increase of mixed receptive fields. This suggested the strengthening of weak afferent input, which might be mediated by sprouting or other mechanisms. Mendell et al. (75) made similar observations when they recorded from

spinocervical tract cells after L7 spared root deafferentation. They also found a loss of responsiveness when peripheral areas supplied by the spared root were stimulated. There was a subsequent recovery of responsiveness owing to an increase in high threshold mechanoreceptive input. Thus although there was recovery, the recovered input was not the same, or in the same proportions, as the original normal input.

Hindlimb Deafferentation

To study the effect of dorsal rhizotomy on loss and recovery of motor function, the dorsal roots L_1–S_2 were cut on one side of the spinal cord (76). Reflex and overground locomotion were studied as in the other models. The initial deficit is more severe than after the other lesions described above. The deafferented limb is not used in locomotion but is dragged behind the animal as it walks on three legs. The descending reflexes, including the scratch and vestibular placing reflexes, are eliminated. All segmental reflex behavior, including crossed reflexes from the contralateral hindlimb, is abolished. Recovery begins soon, on the second postoperative day, and consists of participation of the deafferented limb in the step cycle for overground locomotion. The frequency of stepping is reduced from normal, i.e., the deafferented limb does not step each time the contralateral hindlimb steps. The kinematic pattern is clearly abnormal and the steps of the deafferented limb are sometimes hypermetric and sometimes hypometric. There is also some recovery of the descending scratch and vestibular reflexes during the first postoperative week. During the second postoperative week a marked change is seen in the use of the deafferented hindlimb. The limb can now be used for accurate placement on a narrow (5 cm) runway. Although weight-bearing is somewhat deficient, the animals can place the limbs under the center of gravity and bear weight. Initially there are many errors, but the number of errors decreases and the speed of crossing increases.

In the other two models, hemisection and spared root, the relationship between postural reflex recovery and recovery of locomotion could be examined. Since all afferent input has been eliminated on one side, a similar analysis cannot be carried out in this model. However, it is possible to compare descending control of locomotor recovery and reflex control (Fig. 3). Overground locomotion depends on the presence of descending input: since it is conditioned, it presumably requires input from the brain. Quadrupedal treadmill locomotion requires descending input of the propriospinal system but does not require supraspinal control. Bipedal hindlimb locomotion requires only segmental systems since it recovers after complete spinal transection. When these three types of locomotion are examined after deafferentation, it becomes clear that the recovery is selective. Overground locomotion on the runway begins to recover during the first week. Quadrupedal locomotion on the treadmill begins to recover during the second week and the frequency of stepping of the deafferented limb is permanently reduced. Bipedal locomotion on the treadmill does not recover at all. This implies that activation of the deafferented limb requires descending supraspinal or propriospinal input but that segmental input activated by the contralateral hindlimb is inadequate. Thus recovery of locomotion is mediated by descending systems but not by segmental systems, or segmental systems by themselves cannot mediate locomotion after deafferentation.

This result was surprising considering that the spinal pattern generator for locomotion remained intact. We considered that after deafferentation, the deafferented limb might be dominated by some descending inhibitory system. If this were true, then removal of descending input after recovery from deafferentation might release the deaf-

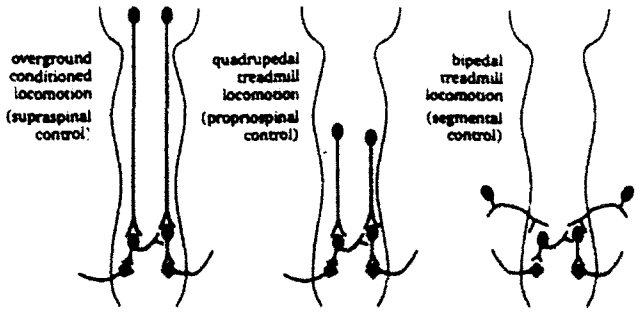

FIG. 3. These diagrams indicate pathways required for the three types of locomotion tested. Conditioned overground locomotion requires the presence of descending supraspinal pathways. Quadrupedal locomotion on a treadmill requires descending propriospinal connections between the forelimb and hindlimb segments of the spinal cord. In contrast, bipedal treadmill locomotion requires only the local spinal pattern generators in the lumbosacral segments.

ferented limb from inhibition and permit the return of bipedal hindlimb stepping. Therefore the spinal cord was transected after the recovery from deafferentation had reached a plateau. There was no subsequent recovery of locomotion in the deafferented limb and even the recovery of locomotion on the other side was slower and less extensive than in otherwise intact spinal cats. An alternative explanation for the results is that there is simply inadequate facilitation, after deafferentation, to activate locomotor circuits on the deafferented side when descending pathways are inactivated by the behavioral conditions (i.e., when bipedal rather than quadrupedal locomotion is tested) or by transection.

These behavioral observations cannot completely define the mechanisms underlying recovery. The anatomical patterns seen after chronic deafferentation are, however, consistent with the pattern of recovery. First, there is a complete recovery of terminal number in major target areas of the dorsal roots (25). Second, when the distribution of staining using a monoclonal antibody specific to dorsal roots is examined, it is clear that the deafferented side remains depleted of dorsal root input and that dorsal roots from the intact side do not invade deafferented regions (30). This is consistent with the observation that recovery does not depend on contralateral systems. Third, an interneuronal system containing substance P and a descending system containing serotonin both exhibit increases in transmitter content in the dorsal horn after deafferentation (11). This is consistent with the idea that descending control is important in mediating recovery of locomotion after deafferentation.

Mechanisms of Sprouting

There are two classes of hypotheses for mechanisms that may regulate sprouting: 1) receptor modification secondary to denervation, which promotes formation and stabilization of new synaptic terminals, and 2) release of growth factors in the environment that can stimulate neurite outgrowth and synaptogenesis. There is as yet relatively little direct evidence that any of these factors contribute to sprouting in vivo.

Receptor Modification

A lesion partially denervates its target neuron and creates vacant synaptic sites, which may evoke denervation supersensitivity on the target neuron. The experiments to relate vacated sites or receptor modification to the formation of new sy-

naptic terminals by intact neurons have not yet been done. There is, however, evidence that dorsal rhizotomy which modifies both the SP and the 5HT projections within the dorsal horn (11,19,21,50–52) is also associated with an increase in the density of tachykinin receptors (77–79) and a down-regulation of 5HT receptors (80). These events may be related to sprouting, as elsewhere in the CNS. The N-methyl-D-aspartate (NMDA) receptor-mediated portion of the excitatory amino acid system, for example, has been shown to play an important role in activity-dependent mechanisms associated with synaptic plasticity and stabilization in other CNS systems (81,82). In the denervated hippocampus, the NMDA receptor density increases transiently, a phenomenon that might promote stabilization of newly formed synapses from sprouting afferents (84). It is therefore of interest both that DRG cells contain excitatory amino acids and that NMDA-binding sites (83) are present in high densities in regions of the dorsal horn where plasticity has been demonstrated. Surprisingly, blockade of the NMDA receptor has been reported to induce sprouting of undamaged dorsal root afferents in adult spinal cord (85). It is likely that new information on the role of receptor changes in sprouting response will be forthcoming.

Growth Factors

Peripheral sprouting and regeneration are known to be regulated by growth factors although the regulation of these two processes may be different (86). There is considerable evidence that polypeptide growth factors are secreted by target or supporting cells, bind to receptors on axons, become internalized, and are transported retrogradely to the cell body where they activate genes whose expression leads to synthesis of proteins necessary for neurite outgrowth (87). Both NGF and insulin-like growth factors promote sprouting from sensory neurons both in vitro and in vivo (88–90), and NGF administration will prevent some effects of peripheral nerve section (91,92). These studies suggest that one stimulus for plasticity at least of DRG neurons is the elimination of NGF, which is normally supplied to the DRG neurons. Synthesis of growth-associated proteins, but not of cytoskeletal elements (92), is stimulated by NGF, and increased synthesis of GAP43 is associated with central sprouting (39, 92). The identification, isolation, and function of growth factors in regulation of central sprouting in vivo remains a challenge.

INTRASPINAL TRANSPLANTS

In the previous sections we have documented the considerable plasticity of the spinal cord after injury in the adult or neonatal mammal. A next step is to attempt to extend or enhance the naturally occurring plasticity of spinal neurons by introducing transplants into damaged spinal cord.

One strategy for promoting recovery after spinal cord injury is to transplant brainstem monoaminergic neurons that can restore function without reconstituting damaged neuronal circuits (93,94). The rationale for this approach is based on the results of experiments suggesting that monoamines administered systemically after spinal cord transection can activate the intrinsic spinal cord circuitry that mediates locomotor (95) or autonomic (96) function. Both brainstem catecholaminergic neurons important for locomotion and serotonergic neurons important for autonomic function have been transplanted into the caudal region of transected spinal cord (97). The transplanted noradrenergic locus coeruleus and serotonergic mesencephalic or medullary raphe neurons extend axons for up to 1–2 cm into host spinal cord (94,98–101), restore levels of neurotransmitter depleted by the transection (102,103), and terminate in the same regions as in normal spinal cord (101–103). Transplanted serotonergic axons

establish synapses on host motoneurons and neurons in the host intermediolateral column that are similar to those formed by brainstem serotoninergic axons in normal spinal cord (102,103). The projections of transplanted locus coeruleus neurons have not yet been studied with the electron microscope.

Both types of transplanted brainstem monoaminergic neurons contribute to recovery in experimental models of spinal cord injury. Rats with spinal cord transections recover reflex ejaculation if they receive transplants of embryonic raphe serotonergic neurons, but rarely if they receive no transplant or a transplant that does not contain serotonergic cells (102,103). Transplanted noradrenergic locus coeruleus neurons are thought to account for the recovery of hindlimb flexion reflexes in rats whose catecholamines have been chemically depleted (104) and for the recovery of reflex stepping activity in rats whose spinal cord has been transected (105). These embryonic transplants therefore contribute to behavioral recovery although they have been placed in the spinal cord caudal to transection and cannot be regulated normally by the host or restore the damaged neuronal circuits. The activation of intrinsic spinal cord networks by the release of transmitter onto or in the vicinity of the normal targets of these neurons appears to be adequate to account for the recovery of these behaviors.

The recovery of other types of behavior lost after spinal cord injury is likely to require more faithful reconstruction of the damaged neuronal circuits. Additional strategies using peripheral nerve grafts or embryonic spinal cord transplants have therefore been developed. Such transplants may contribute to the restoration of function in at least three ways (Fig. 4): 1) by

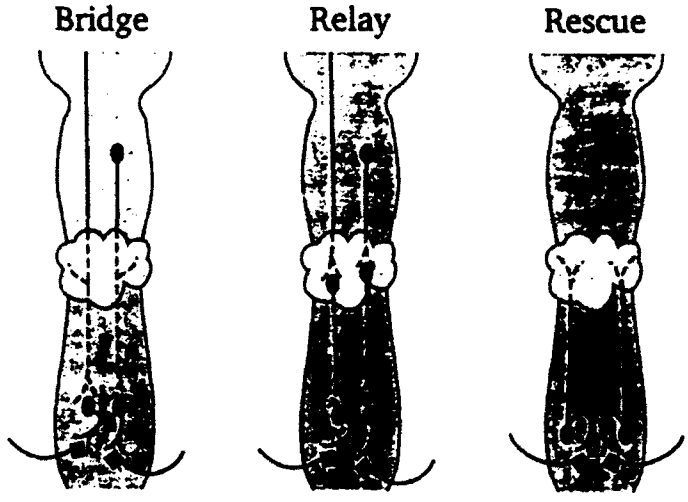

FIG. 4. Diagram illustrating three possible mechanisms by which intraspinal transplants can enhance locomotor function after spinal cord injury. The transplant *(pale area)* is shown at the site of spinal transection. The four neurons in the lower portion of each diagram represent the lumbar spinal pattern generator for locomotion. The vertical lines in the left and middle diagrams represent the axons of supraspinal and propriospinal neurons or of neurons within the transplant and in the third diagram the axons of neurons within the lumbar pattern generator. *Bridge* shows axons of host neurons traversing the transplant. *Relay* shows the establishment of connections within the transplant between the axons of host and transplant neurons. *Rescue* shows the survival of axotomized host pattern generator neurons that would otherwise die. (Reproduced by permission from ref. 30.)

rescuing axotomized neurons that would otherwise die; 2) by serving as a conduit for the regrowth of damaged host axons directly across an area of damage; 3) by serving as a site in which relays are established between neurons in host spinal cord and neurons in the transplant that may project to host neurons.

Segments of peripheral nerve rescue axotomized retinal ganglion cells (106) and when inserted into the spinal cord support the elongation of intraspinal axons (107–109). The axons of DRG neurons and those that originate from neurons whose perikarya are located close to the site of insertion appear favored to grow. The axons of supraspinal neurons that have been injured farther from their perikarya are less likely to project into the grafts (108). CNS axons can grow within the peripheral nerve graft for distances that exceed their normal length, and retinal axons establish synapses on normal target neurons that retain the normal morphological features and activate the target neurons (reviewed in 110). After leaving the peripheral nerve graft, however, the CNS axons show very limited growth within the host parenchyma and terminate within 1–2 mm of the end of the graft (111). Peripheral nerve grafts have contributed a great deal to our understanding of the importance of the neuron's environment for regeneration, but their contribution to functional recovery after spinal cord injury has received little attention.

Eighty to 90% of embryonic spinal cord transplants now survive in the acutely injured spinal cord of adult and newborn rats (112,113) and in the chronically injured spinal cord of adult rats (114). Transplants also survive in the completely severed spinal cord (115,116). Although they lack the characteristic butterfly shape of normal spinal cord gray matter, several morphological features of these transplants encourage the expectation that transplants can contribute to the reconstruction of interrupted neuronal circuits and replace damaged populations of spinal cord neurons. For example, areas develop within transplants that resemble substantia gelatinosa (117), supporting the hypothesis that transplants might function as relays. The astrocytic reaction that develops between transplant and host is interrupted by regions in which the tissues are apposed and processes pass from one to the other (113). Transplants may also reduce the extent of the astrocytic scarring that follows spinal cord injury (114,118), which is thought to represent an obstacle to regeneration.

The connectivity between transplants and adult hosts has been studied by tract tracing and immunocytochemical techniques, and electrophysiological methods are beginning to be employed. Fetal spinal cord neurons transplanted into adult spinal cord form an extensive network of connections with one another; however, few donor neurons extend processes into host spinal cord, and most of these terminate near the interface between transplant and host (119). The number of host spinal neurons that extend processes into transplants is also limited, and the perikarya of most of these are located within 0.5 mm of the interface (119). Only a few corticospinal (120,121) or serotonergic brainstem (113) axons are found within transplants placed into aspiration cavities made in adult spinal cord, and these axons also penetrate only a short distance into the transplants. In adult hosts, therefore, it seems unlikely that transplants can function as conduits that will allow regenerating axons to traverse a region of injury. When transplants are placed into excitotoxic rather than aspiration lesions where cells are killed but axons spared and the observed growth represents axonal sprouting rather than regeneration, central monoaminergic axons grow more robustly than corticospinal or rubrospinal axons (122).

The possibility that embryonic spinal cord transplants might function as the site of relays between sets of injured adult axons has been tested by studying the ability of cut dorsal roots to regenerate into trans-

plants (123). The cut central processes of DRG neurons cannot regenerate into adult spinal cord in the absence of a transplant, but, when provided with a transplant, at least the subset of dorsal roots that contains CGRP grows in sufficient numbers to allow features of their growth to be analyzed quantitatively (123–126). The terminals of regenerated CGRP-containing dorsal roots form synapses with transplant neurons; as in lamina I of normal dorsal horn, most of these are axodendritic and simple and complex synaptic contacts are present in proportions similar to normal (125). Differences between the synapses formed in transplants and normal lamina I are also found. For example, regenerated CGRP-containing axons are significantly more likely to form axoaxonic synapses than normal. Nevertheless, the presence within transplants of regenerated primary afferent synapses with normal features supports the notion that transplants can support or encourage the formation of relays across regions of damaged spinal cord. Our observation that the axons of donor neurons grow into host sciatic nerve at least raises the possibility that transplants can contribute to reestablishing a damaged segmental reflex arc (123).

The possibility that fetal spinal cord transplants might rescue axotomized neurons that would otherwise die was first confirmed in newborn rats (127). Rubrospinal neurons were permanently rescued by transplants of embryonic spinal cord, a normal target of rubrospinal axons, but not by hippocampus, suggesting that survival after injury depended on target-specific factors. We have subsequently found that the neurons of Clarke's nucleus are rescued in newborn rats *not only* by fetal transplants of their normal targets, cerebellum and spinal cord, but also by transplants of embryonic neocortex (128). The axons of Clarke's nucleus neurons do not normally encounter neocortex, which is therefore an inappropriate target for these neurons. This result suggests that the neurons of Clarke's nucleus can be rescued by several different factors or by a single factor that is produced in several regions of the embryonic CNS. This factor has not yet been identified. It appears not to be produced ubiquitously in the fetal CNS, however, because axotomized Clarke's nucleus neurons die in spite of the presence of embryonic striatum transplants (129). Embryonic CNS transplants also rescue Clarke's nucleus neurons after axotomy in adult rats (128), suggesting that transplants can contribute to recovery in adult as well as in newborns.

When placed into the spinal cord of newborn rats, embryonic spinal cord transplants function as conduits that stimulate or allow the axons of supraspinal neurons to grow across the site of injury. The axons of corticospinal neurons (121) and of serotonergic (130) and other brainstem neurons (131) traverse transplants placed in the injured thoracic spinal cord of newborn rats and extend into their normal regions of termination as far caudal as the lower lumbar segments of host spinal cord. In part the greater growth of newborn axons is due to the continued elongation of developing axons that have not reached thoracic levels at the time of transplantation and therefore have not been axotomized. At least some of those axons that reach the lumbar segments, however, were interrupted by the spinal cord lesion and then regenerated (131).

The idea has been tested that the axons that have traversed the site of spinal cord injury and transplantation alter the development or recovery of locomotor function (132). Newborn rats that received subtotal thoracic spinal cord injuries and transplants of embryonic spinal cord were examined with a battery of tests and compared to rats that received lesions but no transplant. Rats with transplants performed better than rats with thoracic spinal cord lesions alone. For example, when examined 8–12 weeks postoperatively, rats with transplants crossed a mesh runway more quickly and made fewer errors in foot placement than the group

with lesion only. They also recovered more quickly from their errors. The results of this study therefore support the notion that axons that traverse the transplant and grow into caudal host spinal cord are responsible for the improved performance. Because the spinal cord lesion was incomplete, however, other mechanisms for the improved performance are possible. One possibility is that the transplants have changed the response to injury of the residual host spinal cord adjacent to the transplant and allowed axons of supraspinal neurons to grow through host spinal cord rather than through the transplant. The axons of corticospinal neurons are known to grow through regions of newborn spinal cord adjacent to injury (133), and transplants of DRG neurons and Schwann cells have been shown to enhance this response (134). Therefore, supraspinal axons that have traversed host spinal cord rather than the transplant may account for the improved locomotor function or may have contributed to the improved performance.

Locomotor function is also being evaluated by Howland in newborn cats that received a transplant into the site of a complete spinal cord transection. These experiments complement those in rat because the spinal cord lesion is complete rather than subtotal, locomotor function can be analyzed in greater detail in the cat than in rat, and because the anatomical pathways that account for various types of locomotor performance are better defined in cat than in rat. Three types of locomotion, which are mediated by three different types of spinal systems, have been analyzed in the cat (reviewed in 30). Hindlimb motion on a moving treadmill (bipedal reflex locomotion) requires only that the spinal pattern generators for each hindlimb and the connections between them remain intact. Reflex locomotion on a treadmill that requires coordination between forelimbs and hindlimbs (quadrupedal reflex locomotion) depends on propriospinal connections between forelimb and hindlimb pattern generators in the cervical and lumbar spinal cord as well as on the pattern generators themselves. Conditioned (voluntary) overground locomotion for a food reward depends on intact pathways descending from brain as well as on intact segmental and intersegmental connections. In cats with thoracic spinal cord transections that have received a transplant on the day after birth, performance of quadrupedal reflex locomotion will, therefore, suggest that propriospinal connections have grown across the transplant either directly or via relays. In these animals, the presence of conditioned overground locomotion will suggest the growth of axons with perikarya in the brain.

The locomotor function of two cats that received transplants of E26 spinal cord into T12 transections on the day after birth has been examined for periods of 6 weeks and 5 months (115,135). These cats were compared to two cats with transections on the day after birth that did not receive transplants. Both groups developed quadrupedal locomotion in addition to bipedal locomotion, but the group with transplants achieved overground locomotion approximately 6 weeks earlier than the group with transection alone. The preliminary results also indicate that the performance of animals with transplants is far superior to those with transection alone in the ability of the animals to support their weight, to maintain postural stability, and to coordinate the movement of forelimbs and hindlimbs. Even in animals with transplants, however, the coordination is only sometimes similar to that of normal cats, and overground locomotion is abnormal. The postural stability of the hindlimbs, for example, is impaired, the step cycle is prolonged, and the normal 1:1 pairing of forelimb and hindlimb step cycles is inconsistent. In both cats that received transplants and were studied behaviorally, histological evaluation revealed transplants to be present.

Preliminary studies are also being carried

out to identify the anatomical connections in these cats (135). One animal that received a transplant of E21 spinal cord has so far been studied. Descending serotonergic and noradrenergic axons whose cell bodies are in the brainstem grow extensively in this transplant and enter host spinal cord caudal to the transplant. Serotonergic axons grow as far caudally as the host L6 segment. Regenerated CGRP-immunoreactive host dorsal roots and substance P-containing processes that arise from multiple sources are also found throughout the transplants, where they are accumulated in some areas that resemble the substantia gelatinosa and superficial dorsal horn of normal spinal cord. These preliminary results indicate that transplants enhance the development of locomotor function in newborn cats with complete spinal cord transections and suggest that this effect is mediated by descending axons that grow into the transplants. Whether these axons alone account for the enhanced locomotor function remains to be determined.

SUMMARY

Plasticity of undamaged projections in the adult mammalian spinal cord has been documented many times and by a number of different methods, including degeneration techniques, tract tracing, immunocytochemistry, and quantitative electron microscopy.

Sprouting in the adult appears to be spatially limited and regulated rather than random. When damage is made in the neonate, the amount of sprouting or the distance over which sprouting takes place is usually, but not always, greater than in the adult. In some paradigms, the sprouting has been associated with recovery of motor behavior, and specific systems that sprout can be related to recovery of specific functions. This normally occurring plasticity may be enhanced by the use of embryonic CNS or peripheral nerve transplants; furthermore, transplants can encourage axonal growth. For example, cut dorsal root fibers will grow into a spinal cord transplant and make connections that appear morphologically similar to normal. Descending projections can also grow into the transplant and, in the neonate, these axons grow not only into the transplants but also extend caudal to the transplants and into the host spinal cord. These animals display greater and qualitatively different recovery of function than those with lesions but not transplants. At the present time, the mechanisms underlying transplant-mediated recovery are unclear. For example, we do not know whether transplanted tissue acts as a bridge or as a relay in order to mediate recovery. The anatomical and functional analyses that have been carried out in sprouting systems have not yet been applied to transplants. Such studies will undoubtedly contribute to our understanding of the mechanisms by which transplants can contribute to enhanced recovery of function after spinal injury.

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CHAPTER 21

Transplantation of fetal spinal cord tissue into acute and chronic hemisection and contusion lesions of the adult rat spinal cord

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Introduction

Demonstration that grafts of fetal central nervous system (CNS) tissue can reverse deficits associated with a variety of brain disorders and lesions has led to several recent examinations of whether embryonic neural tissue can also be used to promote functional recovery in the injured spinal cord. In this regard, the emphasis of intraspinal transplantation is usually placed upon the potential of fetal cell implants to restore locomotion. It is also of great interest, however, to define whether such transplants can ameliorate any of the other frequent complications of spinal cord trauma, such as spasticity and chronic pain.

While from this perspective of functional repair it is conceivable that some deficits may be reduced by restoring appropriate levels of neurotransmitters in neuronal pools below the lesion, the recovery of other functions might very well require considerable remodeling of synaptic circuitries in the host spinal cord. Since the latter would demand the formation of extensive afferent and efferent axonal projections, it seems that fundamental to the restoration of spinal cord function via transplantation is the need to establish a suitable morphological substratum whereby donor neurons can ultimately influence the host CNS. In particular, consideration should be given to whether the anatomical setting associated with various types of spinal cord injury, including those resembling lesions in the human, can influence the feasibility of intraspinal transplantation and the relative extent to which neural interconnections are formed. Along these lines, the present chapter summarizes observations from our recent neuroanatomical and immunocytochemical studies in which we have examined: (1) the pattern of axonal connectivity established with fetal homotopic (i.e. spinal cord) grafts into the acutely injured spinal cord, (2) the influence of an existing histopathology on survival of these transplants and their integration with the host spinal cord in chronic lesions, and (3) the feasibility of transplantation into chronic, contusion lesions.

Neuroanatomical Studies of Fetal CNS Grafts in Acute Spinal Cord Lesions

Two basic intraspinal grafting strategies are currently being investigated for their potential to promote functional repair of the damaged spinal cord (Nornes et al., 1984; Reier, 1985). The first entails the injection of dissociated fetal cells, enriched with selected neuronal populations of supraspinal origin, into the parenchyma of the spinal cord caudal to the site of injury (for details, see Chapters 00, this volume and Björklund et al., 1983, 1986; Buchanan and Nornes, 1986; Privat et al., 1986). The second approach, which is the focus of this chapter, involves the implantation of tissue into the lesion site itself. In theory, this strategy could

Chapters by: → Privat
→ Nornes

be useful in establishing a more favorable cellular microenvironment for axonal elongation between separated segments of the spinal cord through the development of a tissue bridge. It is also conceivable that fetal neural tissue grafts at the site of injury could stimulate functional recovery by forming a novel spino-spinal relay network between the rostral and caudal stumps (Fig. 1).

We originally reported long-term survival and some organotypic differentiation of fetal homotopic tissue, introduced as whole tissue segments, in acute hemisection cavities of the adult spinal cord (Reier, 1985; Reier et al., 1985, 1986a,b). More recently, we have also shown that it is possible to reconstruct large intraspinal defects with suspensions of dissociated fetal spinal cord cells (Houlé and Reier, 1986). In most cases, both types of graft spanned the length (up to 6 mm) of the lesion and partially fused with the injured rostral and caudal surfaces of the recipient spinal cord. We subsequently began charting projections developed between fetal intraspinal grafts (one to four months post-transplantation) and the adult host CNS ~~as identified~~ with retrograde and anterograde horseradish peroxidase (HRP) tracing methods (Reier et al., 1986a,b).

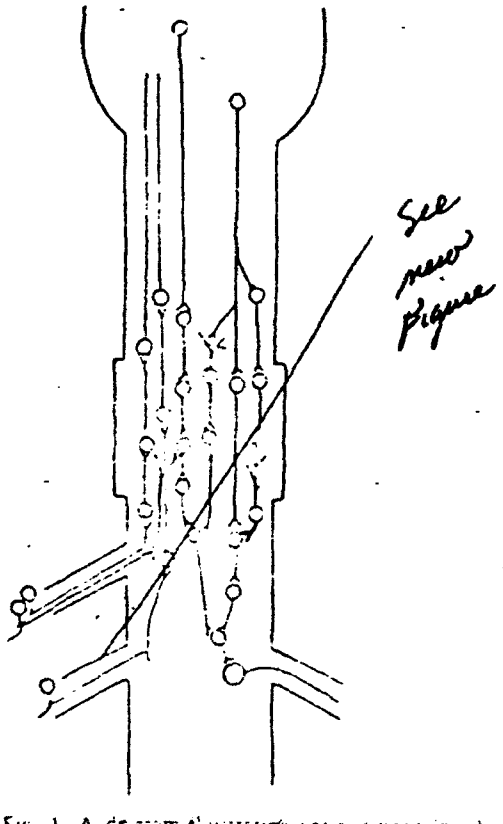

Fig. 1. A diagram illustrating various ways in which an intraspinal graft, introduced at the site of injury, may establish a novel spino-spinal relay for conduction of motor and sensory information past the lesion. The various configurations of neural circuitries and the relative length of axonal projections into and out of the graft are based upon evidence obtained in the neuroanatomical studies described in the text.

Injection of the tracer into either the rostral or caudal segments of the host spinal cord at distances of 5 – 7 mm from the host-graft interface resulted in retrograde labeling of donor neurons: no labeling was observed, however, following tracer injections at greater distances from the graft site. Taking into account some diffusion of HRP, we estimate that the maximum outgrowth range of most axons from fetal spinal cord neurons into the host spinal cord is on the order of 3 – 5 mm. Our findings thus far indicate considerable variability in the number of labeled donor cells from one recipient to another. On the other hand, there appears to be a consistent pattern in the distribution of HRP-containing neurons in these homotopic graft as the majority are located near the host-grafts interface with much smaller numbers of labeled cells being present at the opposite pole of the grafts.

Injections of HRP into the host spinal cord have also demonstrated some anterogradely labeled axons projecting for short distances into grafts. This observation was consistent with the fact that when HRP was injected into transplants in other experiments, some retrogradely labeled host neurons were present in the intermediate gray regions of adjacent spinal cord segments. It is also worth noting that with small injections into the larger grafts, widespread donor neuron labeling was seen beyond any detectable zone of tracer spread. This indicates considerable intragraft connectivity.

While in this second group of experiments, labeled cells were observed in the host CNS, none were found beyond 5 mm from the host-graft junction, suggesting an absence of any long propriospinal or supraspinal input. Other experiments, however, involving immunocytochemistry revealed some growth of serotoninergic (5-

Per letter from Mr. Bruker, Figure 1 has been modified (enclosed). Figure legend has also been rewritten.

HT) fibers into the grafts; these 5-HT-like immunoreactive fibers usually terminated within a relatively short distance beyond the host-graft interface. Thus, failure to obtain retrograde labeling of cells in the host brainstem following HRP injections of the transplant may have been due to the effective injection site (i.e. toward the center of the grafts to avoid spread of tracer) being out of register with the terminal fields of these axons. This may also explain why only a small number of host intraspinal neurons was labeled following injection of the homotopic grafts.

The most robust innervation of intraspinal grafts that we have observed thus far has derived from host primary afferent fibers. As shown by Tessler et al. elsewhere in this volume (see Chapter 27), insertion of dorsal roots directly into intraspinal transplants can result in an extensive ingrowth of sensory axons. Using antibody to CGRP (calcitonin gene-related peptide) we have also observed that primary afferent fibers can extend directly from the host spinal cord into either homotopic or heterotopic grafts (Fig. 2) placed near the thoraco-lumbar junction. The ingrowth of primary afferent fibers is of special interest given that areas resembling the superficial dorsal horn – a target for CGRP-immunoreactive fibers – frequently appear within homotopic transplants (Reier et al., 1986a,b).

Because of difficulties frequently encountered with tracer diffusion either following injection of the transplant or of the host spinal cord near the host-graft interface, we still regard these findings as being an overly conservative estimate of the degree of connectivity achieved under these conditions. For example, silver-stained or plastic thick sections taken from regions where a confluent neuropil is established between host and graft sug-

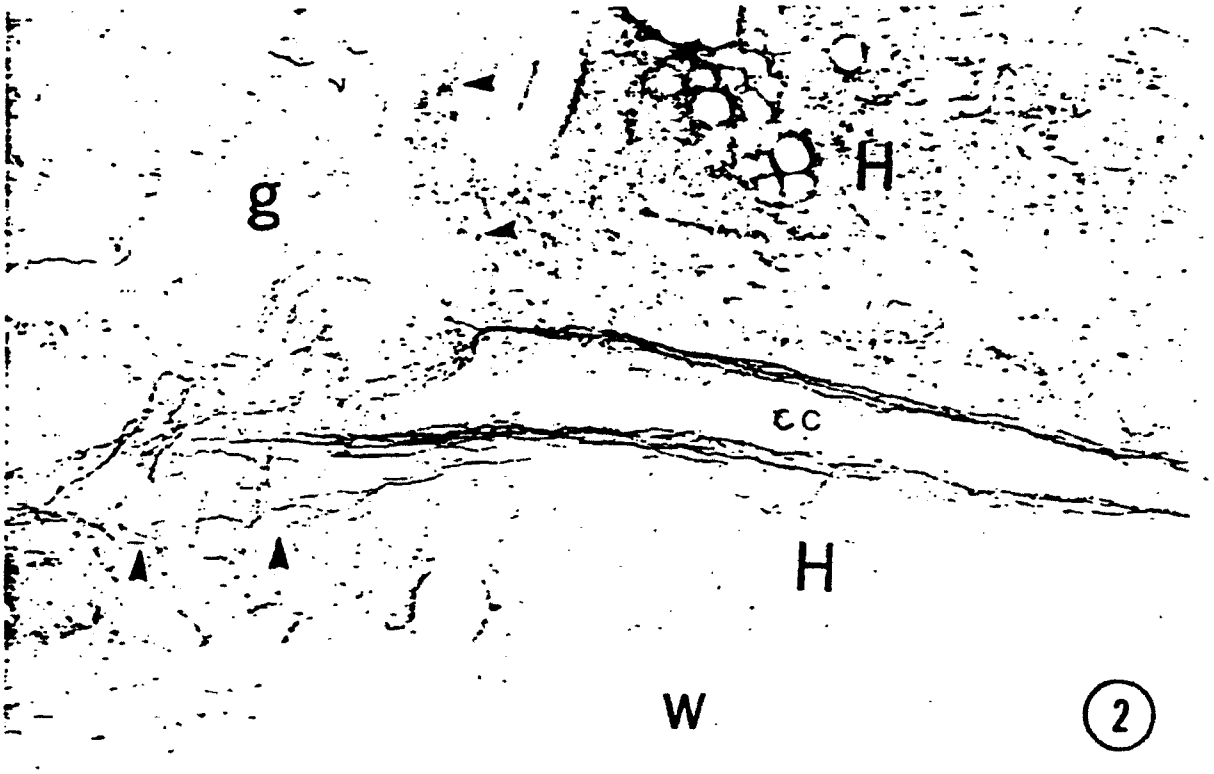

Fig. 1. A diagram illustrating various ways in which an intraspinal graft, introduced at the site of injury, may establish a novel spinospinal relay for conduction of motor and sensory information past the lesion. The various configurations of neural circuitries and the relative length of axonal projections into and out of the graft are based upon evidence obtained in the neuroanatomical studies described in the text.

gest considerable neuritic growth between the two areas. In fact, some sections have even indicated considerable extension of dendrites across these interfaces. This has been further supported more recently by experiments involving retrograde labeling of host motoneurons with cholera toxin-conjugated HRP.

Despite some of the technical problems mentioned which we are now trying to resolve with other axonal tracing methods, our results provide some useful information concerning the pattern of connectivity that can be achieved in the acutely injured spinal cord. While there is presently no evidence in support of homotopic grafts serving as a bridge for axonal elongation in adult recipients, our initial neuroanatomical findings favor the potential of fetal CNS tissue grafts for developing a relay circuit at the injury site. As summarized in Fig. 1, our evidence suggests that this relay can assume a variety of configurations involving mono-, di- or polysynaptic relationships within the transplant.

One of the major challenges that confronts this approach to spinal cord repair, of course, is to determine how descending influences which may be transmitted through this relay can then be conducted to affected motoneuron pools. In the absence of long-tract regrowth, this would obviously require activation of intersegmental circuitries caudal to the injury (Fig. 1). Furthermore, apart from some serotoninergic input to homotopic transplants, it is still unknown whether any other descending systems innervate these grafts. It is interesting, however, that other studies in this laboratory (Jakeman and Reier, 1987) have indicated that many severed corticospinal fibers and their collateral projections into gray matter remain distributed along the host-graft interface. By virtue of an outgrowth of dendrites from donor neurons

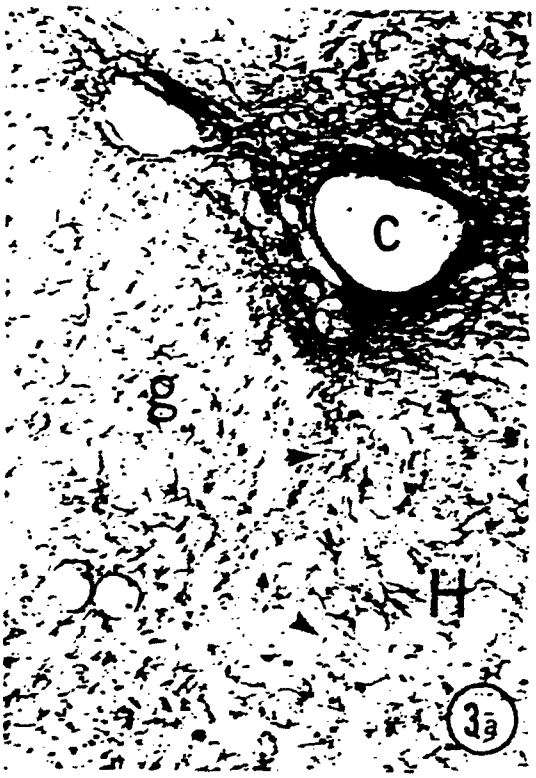

Fig. 3. a. A horizontal section, stained with antiserum to glial fibrillary acidic protein, showing approximation of a fetal spinal cord graft (g) with the dorsal gray matter of the host spinal cord (H). Note the absence of gliosis along the interface established with host gray matter (arrowheads). A pronounced gliosis is seen in the corticospinal tract in which a small cyst (C) is present. b. A more ventral horizontal section from the same specimen now shows a well-defined glial scar between host (H) and graft (g)

near this interface, it seems possible that some form of neural interaction could be achieved even in the absence of regeneration.

Glial Interfaces at host-graft junctions following transplantation into acute and chronic lesions

We and others (Nornes et al, 1983, Das, 1983) have observed sites of excellent fusion of host and graft tissue (Fig. 3a); however this is a highly variable feature. For example, in the same specimen one can observe many regions in which a graft is separated from host tissue by microcysts or astroglial scars. This is especially prevalent in regions of degenerated white matter (Fig. 3a), although the same is seen in many areas where the grafts approximate (but do not fuse with) host gray matter (Fig. 3b).

As discussed in recent reviews (Reier et al, 1983a; Reier, 1986; Reier and Houlé, 1987), a considerable body of evidence suggests that glial scars are incompatible with sustained axonal elongation in the mature CNS, although the mechanism underlying this inhibitory effect has still not been identified. The ability of glial scars to compromise the outgrowth of axons from fetal grafts has also been indicated in our intraspinal transplantation studies. For example, we have observed many instances in which axons from donor neurons are apparently deflected back into the transplant upon reaching dense gliotic areas along the host-graft interface.

In view of these observations, the extent to which connectivity can be achieved between host and graft appears to be at least partly dependent upon the extent of glial reactivity at the transplantation site. From a more clinical perspective, this raises some questions regarding the feasibility of transplantation into ~~chronic~~ lesions characterized by a long-standing histopathology that can include extensive gliosis.

In the only published study in which a delay prior to intraspinal transplantation of fetal CNS tissue was attempted, Nornes et al. (1983) reported poor viability of fetal brainstem tissue. A significant difficulty in actually placing the graft into the original lesion site was indicated which could have contributed to these results. It should also be noted that their choice of implantation site, viz. a cavity in what was the central gray matter of the spinal cord, was surrounded by white matter which provided minimal access to blood vessels required for vascularization of the grafts. Thus, it is likely that these initial attempts failed for purely technical reasons.

In a more recent study (Houlé and Reier, 1988) successful intraspinal transplantation of fetal spinal cord tissue was achieved with two to seven delays between the initial hemisection lesion and grafting. Therefore, the advanced pathology of the chronically injured spinal cord does not seem to represent an unfavorable milieu in terms of the survival, growth, and differentiation of fetal spinal cord tissue as many of the features of grafts in the chronic spinal lesion paralleled those seen in grafts placed into acute injuries. In addition, many sites of confluent host-graft neuropil were observed, and some evidence of connectivity, similar to that described above, was also obtained. The fact that sites of direct graft and host fusion could be routinely identified, despite the presence of an existing dense glial scar at the time of transplantation, raises the possibility that fetal CNS tissue has a capacity for stimulating a partial regression of an established glial scar. A more practical benefit of these findings is that they provide an insight related to the injured spinal cord under conditions that simulate the most likely clinical circumstances under which potential intraspinal transplantation can be envisioned.

Transplantation into the contused spinal cord

The traditional approach to spinal cord regeneration research over the years has relied upon the use of a complete transection model. Although erroneous interpretation of data has arisen as a result of poorly documented and incomplete lesions, this approach nonetheless has offered the benefit of providing a reproducible injury with predictable behavioral deficits. However, one of the disadvantages of this lesion is that it fails to reproduce the type of injury commonly encountered in the clinical setting. Except in cases of penetrating missile or stab wounds, most instances of human spinal cord damage involve partial destruction of cord tissue and incomplete disruption of anatomical continuity as a result of blunt trauma and frequently

associated fractures or dislocation of the vertebral column.

In 1911, Allen described an experimental model of contusion injury having a pathology closely resembling that seen after blunt trauma to the human spinal cord. Although extensively used over the years, this lesion approach has been criticized for its lack of predictability and reproducibility. The value of the Allen model, however, has never been totally discredited, and in the last few years modifications have been made to this approach which are now yielding more reliable results in terms of reproducible lesions and predicatable short- and long-term behavioral outcomes (e.g. Wrathall et al., 1985; Gale et al., 1985; Bresnahan et al., 1987; Somerson and Stokes, 1987). These recent developments provide an excellent opportunity for testing different transplantation strategies for stimulating the recovery of function during both acute and chronic phases.

Although the success of transplantation has frequently led to the assumption that graft survival can be obtained under virtually any condition, each lesion presents its own set of unique circumstances. Thus, our first approach to transplantation in the contused spinal cord was to determine to what extent homotopic graft survival could be achieved after transplantation into severely contused spinal cords at two to fourteen months post-injury (Wirialski et al., 1987). It was found that over 90% of the grafts survived and filled cavities measuring up to 7 mm in length (Fig. 4). In most cases, the grafts were closely approximated with the rostral and caudal ends of the host spinal cord. While a dense matrix of gliosis often intervened, some areas of apposition were observed at which minimal scar formation was indicated. In plastic thick sections numerous neuritic processes traversed the interface, and immunocytochemistry showed that some 5-HT-like immunoreactive fibers had entered the transplant and extended for a distance of 2 mm.

Conclusion

Together, these findings have indicated that it is feasible to transplant embryonic CNS tissue, as exemplified by homotopic grafts, into various lesions of the adult spinal cord, including those which may ultimately shed light on the potential clinical application of intraspinal transplantation. These studies have also established a useful neuroanatomical framework for physiological and behavioral tests of the functional impact of these grafts in acute and chronic hemisected and contused spinal cords.

It should be stressed, however, that while evidence exists for some axonal connectivity between host and graft, it is still uncertain that an optimal setting has been established under any of these lesion conditions, as indicated in part by the variable glial responses seen in individual transplant recipients. In addition, very little is known either about the neuronal growth properties or functional organization of the chronically injured, as well as contused, spinal cord. Therefore, any accurate realization of the potential of fetal CNS grafts to restore function in the injured spinal

Fig. 4. An osmicated sagittal section showing a 7-mm long fetal spinal cord graft two months after being placed into a chronic contu[illegible]

cord may still be far removed. Nevertheless, intraspinal transplantation has begun to stimulate new avenues of investigation which will undoubtedly facilitate a more in-depth understanding of the complex biology of the injured spinal cord.

Acknowledgments

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Fig. 1. A diagram illustrating various ways in which an intraspinal graft (shaded area), introduced at the site injury, may establish a novel spino-spinal relay for conduction of motor and sensory information past the lesion. For orientation, the top of the figure represents cortical and brainstem regions. The various configurations of neural circuitries and the relative length of axonal projections into and out of the graft are based upon evidence obtained in the neuroanatomical studies described in the text. Note that the possibility of dendritic sprouting by host (hatched circle) and donor (solid circle) is also indicated. MN = motoneuron; DRG = dorsal root ganglia.

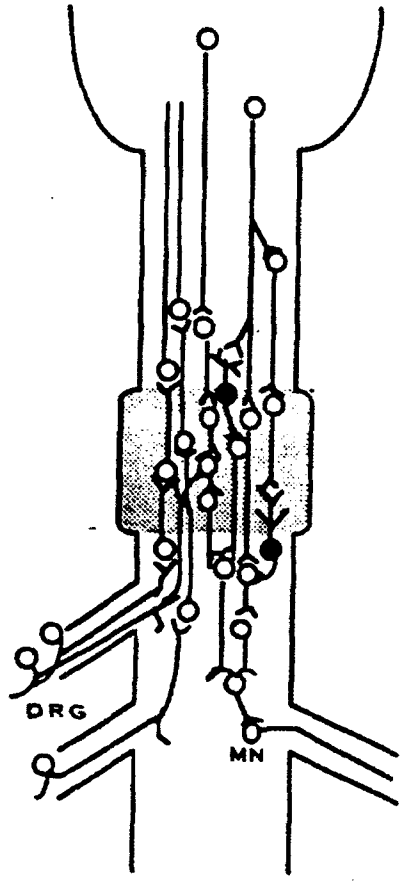

Fig. 1

Current Opinion in NEUROLOGY NEUROSURGERY

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Transplantation

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Transplants of embryonic central nervous system (CNS) tissue send processes into host brain or spinal cord and provide a permissive environment for the ingrowth of host fibres. The motivation for this line of research is the hope that the connections established between neurons in the transplant and host will improve host function. New approaches include genetic modification of transplants to increase their survival and to increase the biologically active molecules available to the host.

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Introduction

Transplants of embryonic central nervous system (CNS) tissue have been used for many years in experiments aiming to clarify the mechanisms that underlie axon outgrowth and regeneration. Many clinicians first became aware of the therapeutic applications of transplants after the publication of reports that autografts of adrenal medulla placed in the caudate nucleus produced improvement in the motor deficits of patients with Parkinson's disease. Since then more than 250 adrenal medullary autografts and a number of grafts of fetal mesencephalon into host striatum have been performed [1]. Several reports describing the results of these procedures have been presented in the past year [2•,3•]. One review has criticized both the experiments that led to the use of transplants as therapy for Parkinson's disease and the notion that transplants were responsible for the modest improvement observed in some patients [4•]. The author of a more measured review, who included an optimistic assessment of the future of transplant therapy, concluded that transplants had harmed more patients than they had helped [5•]. The expectation that transplants would become part of the routine therapy for Parkinson's disease therefore remains to be fulfilled.

Transplants nevertheless have been reported to produce improvement in experimental models not only of Parkinson's disease, but also of Huntington's disease, Alzheimer's disease, spinal cord injury, and chronic pain. They remain of considerable interest both for their potential use in the clinic and for the insight that they can provide into the mechanisms underlying regeneration and recovery of function. In the present selective review we discuss evidence published in the past year that transplants contribute to recovery, consider the mechanisms by which transplants act, and present some new approaches to the experimental and clinical use of transplants.

Connectivity of grafted and host neurons

The behavioral effects produced by transplants in laboratory models of human diseases may be mediated by a number of different mechanisms, including the release of neurotransmitter in the vicinity of the normal target or into a ventricle, the prevention of neuron death, the stimulation of collateral sprouting by host axons, as well as the re-establishment of interrupted connections between neurons [6•]. The ability of transplants to restore behaviors that depend on the proper functioning of pathways that communicate by point-to-point connections will depend at least in part on the extent to which neurons in the transplant re-establish normal connections.

The extent to which the axons of donor neurons project into host tissue may depend on many factors, including the type of transplanted neuron, the location of the transplant, and the type of injury. Embryonic spinal cord neurons transplanted into aspiration cavities in the spinal cord of adult rats establish an extensive network of connections within the transplant [7•]. Few donor neurons send projections into host spinal cord, however, and most of these terminate near the interface between the transplant and the host, although some extend up to 5 mm into host spinal cord. In intact hosts and in rats whose intrinsic noradrenergic neuron terminals in the hippocampus have been destroyed by 6-hydroxydopamine (6-OHDA) injection, embryonic noradrenergic neurons of the locus coeruleus send large numbers of axons into the hippocampus of adult host rats, where they establish synaptic contacts with host neurons [8•]. Cell suspensions of embryonic rat striatal tissue also show robust ingrowth into the host globus pallidus when the transplants are placed into a lesion created by the injection of an excitotoxin [9•]. The axons of the grafted neurons extend along the normal pathway of striatal axons in the internal capsule and form synapses with target

Abbreviations

CGRP—calcitonin gene-related peptide; **CNS**—central nervous system; **DRG**—dorsal root ganglion; **NGF**—nerve growth factor; **6-OHDA**—6-hydroxydopamine; **TH**—tyrosine hydroxylase.

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neurons that resemble those formed normally by striatal neurons in the globus pallidus. These results suggest that the cues that direct axonal outgrowth and the signals that lead to the formation of synapses persist or are reactivated in the denervated adult host and that these signals can be recognized by the axons of the transplanted neurons. Results obtained when embryonic mouse retinas are grafted into the midbrain of newborn rats indicate that these cues are multiple and are likely to originate in both the immediate environment of the transplanted neurons and from the target [10•]. The outgrowth of the optic axons is not random and is directed towards their normal target in the superior colliculus, but the pathway followed by the axons depends on their initial location. Axons from retinas placed close to the surface of the midbrain grow along the glia limitans, whereas axons from those placed within the midbrain parenchyma grow through the neuropil to reach the overlying superior colliculus. Additional insight into the mechanisms by which the axons of donor neurons are guided to their targets within the host is provided by the observation that axons of embryonic hypothalamic neurons transplanted into the third ventricle of hypogonadal mice grow into the median eminence and restore gonadal function even though neurons along their path have been destroyed [11•]. These results suggest that directional cues in this system are likely to originate in the host ependyma, glia, or target neurons.

A particularly striking example of the persistence of directional cues and the specific reformation of a damaged pathway is provided by experiments in which human fetal forebrain neurons are transplanted into the excitotoxically injured basal ganglia of adult rats [12••]. The transplanted axons of these embryonic neurons elongate for distances of at least 20 mm along myelinated fiber tracts and into their normal target areas in substantia nigra, pontine nuclei, and cervical spinal cord. The lengthy outgrowth may be attributable to the prolonged developmental timetable of human neurons that allows the growth-promoting features of the neurons to continue to over-ride the inhibition of the adult rat CNS for longer periods or to the greater length that human axons normally achieve. The axons appear to recognize specific cues that persist for at least 1 year after the injury but are not detected by transplanted human hindbrain neurons which show very little process outgrowth [12••].

The ability of embryonic transplants to support or enhance the growth of injured adult axons is relatively modest but ingrowth does reflect the intrinsic capacity of the injured neurons to regenerate. Monoaminergic axons, for example, grow more robustly into transplants of fetal thalamic cells than do somatosensory afferent axons, suggesting that neurons whose axons terminate diffusely have less stringent growth requirements or a more vigorous capacity for growth than neurons whose axons terminate in precise portions of specific nuclei [13•]. Central monoaminergic axons also grow more extensively than corticospinal or rubrospinal axons into intraspinal embryonic spinal cord transplants [14•]. Intraspinal axons project to only a limited extent into transplants of fetal spinal cord and the perikarya of those few axons that regenerate are for the most part within 0.5 mm of the graft [7•].

The axons of dorsal root ganglion (DRG) neurons terminate in very precisely specified regions of the spinal cord or brainstem, but they are nevertheless capable of elongating into intraspinal transplants of embryonic spinal cord, whether the grafts are placed in an excitotoxic lesion [14•] or aspiration cavity [7•]. Subsets of DRG neurons have been distinguished on the basis of several criteria, including their light microscopic appearance and their immunoreactivity for various neuropeptides [15,16]. It has not yet been determined whether all subsets of DRG neurons, as determined by size or peptide content, are capable of regenerating into transplants or whether this capacity differs among the populations of neurons that constitute the DRG. The axons of some DRG neurons retain the ability to regenerate for at least 4 weeks after injury [17•]. Small neurons are present in greater numbers than would be predicted from the proportion that they represent among the entire population of DRG neurons normally [17•]. This result suggests that small DRG neurons have a greater capacity for regeneration after chronic injury than large DRG neurons. The subset of DRG neurons that are immunoreactive for calcitonin gene-related peptide (CGRP) are capable of regenerating axons into intraspinal embryonic transplants not only of spinal cord but also of regions of embryonic brain placed into spinal cord. In transplants of spinal cord, however, both the area occupied by these regenerated axons and the number of synapses formed is greater than in transplants of brain [18•]. This result suggests that transplants support or enhance the regeneration of cut DRG axons by providing conditions that are probably multiple and relatively non-specific. The normal target of these axons provides additional, more specific cues that determine the final distribution of the regenerated axons and enables them to establish synapses. These synapses retain several, but not all, of the features of the synapses formed by CGRP–containing primary afferent axons in the dorsal horn of normal spinal cord, suggesting that regenerating axons retain the ability to recognize at least some of the cues that direct growth and synapse formation during development [19•]. The specificity with which regenerating axons can grow into a transplant is strikingly illustrated by the observation that the axons of adult host dentate granule cells innervate transplanted CA3 pyramidal cells in embryonic hippocampal transplants rather than CA1 cells and that the innervation terminates on the normal regions of the dendritic tree [20•].

The growth of newborn host axons into embryonic CNS transplants exceeds that of adult host axons, and the axons of newborn supraspinal neurons can traverse embryonic spinal cord transplants placed in thoracic segments and terminate in regions of host spinal cord many segments caudal to the transplants [21]. The more extensive growth of newborn host axons may be caused in part by the continued elongation of developing axons that have not reached thoracic levels at the time of transplantation and therefore have not been axotomized. It is, however,

clear that in newborns with fetal spinal cord transplants, injured, regenerating axons as well as late developing, uninjured axons are among those that reach lumbar segments, showing that the capacity for regeneration in newborn axons exceeds that of young adult axons [22•].

Restoration of function

Potential mechanisms by which transplants can restore function include stimulating collateral sprouting of host neurons or inducing host neurons to increase their synthesis of neurotransmitters. It is difficult to distinguish between these two mechanisms. An example of recovery mediated by host axon sprouting has been reported in monkeys who received transplants of fetal mesencephalon into the caudate nucleus following a unilateral injection of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which destroys the neurons of the ipsilateral substantia nigra [23•]. Recovery as assessed by volitional arm use and loss of asymmetrical turning to apomorphine injection was related to increased staining of host tyrosine hydroxylase (TH) positive (presumably dopaminergic) fibers rather than the ingrowth of transplant axons into the denervated host caudate nucleus. The brains of several patients who had received adrenal medulla autografts to the caudate nucleus for Parkinson's disease 6 weeks to 30 months previously also revealed increased staining of host TH-containing fibers proximal to the graft site although few, if any, adrenal cells survived transplantation [24•,25•]. One of these patients was thought to have benefited at least temporarily from the procedure, and it is possible that sprouting of host axons stimulated by the transplant or the transplantation procedure accounted for the recovery.

Several systems have been studied in which transplant-mediated recovery is related to the capacity of the transplants to restore damaged neuronal circuitry. A particularly striking example is provided by the correlation between the degree to which a transplanted retina innervates its normal target in the host olivary pretectal nucleus and mediates the recovery of a pupillary light reflex [26••]. In another example, newborn rats that received transplants of embryonic spinal cord into a subtotal lesion of the thoracic spinal cord performed better when examined with several tests of locomotor function than littermates with a thoracic cord lesion but no transplant [27••]. These behavioral results together with anatomical studies showing that transplants can serve as a conduit for the growth of supraspinal axons into caudal segments of spinal cord [21] suggest that the axons elongating into the transplant are contributing to the improved behavioral performance. Indirect evidence consistent with dependence of recovery on at least partial restoration of damaged circuitry has been shown by studies of rats with unilateral 6-OHDA lesions of the nigrostriatal pathway [28•]. Animals that received transplants of fetal mesencephalon into the denerved striatum recovered from several behavioral deficits whereas rats that received only systemic administration of levodopa and carbidopa did not, suggesting that replenishing the depleted neurotransmitter alone did not suffice to produce recovery. Fetal mesencephalic grafts, however, do not reverse all of the behavioral deficits produced by unilateral or bilateral denervation of the striatum [29]. Incomplete recovery in these rats is consistent with the results of electrophysiological studies indicating that transplants only incompletely restore damaged neural circuits. Although grafted neurons receive input from neurons in the host neocortex or striatum and may be regulated by this input, the neurons in these grafts and/or the host afferent projections to these neurons remain immature for months after transplantation [30•]. If tested in the early months after transplantation, behaviors that require mature functioning of mesencephalic neurons and their connections would therefore not recover. Incomplete restoration of normal circuitry has also been demonstarted in an experimental system that has been considered to be a model for Huntington's disease. Intracellular recording from neostriatal spiny neurons grafted into an excitotoxic lesion in the striatum of adult rats showed functional afferent connections from host neocortex and thalamus as is characteristic of normal striatal neurons, but afferent input to the grafts and the amount of input converging from different sources were reduced [31•]

New approaches

Several strategies have been employed to improve the survival of grafted and injured neurons and to increase outgrowth from the transplants and axotomized host cells. One approach toward improving cell survival and fiber growth from the transplant has been to co-graft embryonic tissue along with its normal target. When embryonic mesencephalon and striatum are transplanted into the striatum of rats with unilateral 6-OHDA lesions of a nigrostriatal pathway, TH-containing neurons appear to be larger and fiber outgrowth into the host striatum more extensive than from single mesencephalic grafts, and the co-grafts produce more improvement on an amphetamine rotation test [32•]. A similar approach has been to combine a transplant with an infusion of a neurotrophic factor. One patient with Parkinson's disease who received an intraputaminal autograft of adrenal medulla and an infusion of nerve growth factor (NGF) into the graft site showed modest improvement in selected measures of locomotor performance and suffered no complications from the administration of mouse NGF [33•].

Combined therapies have also been used to increase the survival and neurite outgrowth of injured host neurons. The combination of a fetal hippocampus transplant and NGF infusion after unilateral fimbria-fornix section produced a greater increase in the number of septal nucleus neurons that contained choline acetyltransferase immunoreactivity than either a transplant or NGF infusion alone [34•]. The combined treatment also stimulated more extensive regeneration of septal axons into the hippocampus than either treatment alone, encourag-

ing the notion that more injured cells can be salvaged and circuits more successfully reconstructed by a combination of therapies.

Another approach toward encouraging survival and regeneration from host neurons has been through the development of new methods for delivering identified neurotrophic factors and neurotransmitters. To protect against rejection when transplants are performed across species, dopamine-secreting PC12 cells were encapsulated in an acrylic copolymer that allowed TH-containing cells to survive *in vivo* for at least 12 weeks and to release dopamine *in vitro* for at least 6 months [35••]. Tumor cell lines genetically modified to produce NGF or dopamine have also served as grafts, but their usefulness as transplants requires that the peptides continue to be synthesized after transplantation and that the cell lines lose their tumorigenicity. PC12 cells modified to produce NGF did not form tumors following transplantation into mouse brain, but they lost their neuronal morphology, survived poorly, and did not synthesize TH [36•]. A temperature-sensitive line of neuroblastoma cells that at non-permissive temperatures resembles neurons and synthesizes NGF was more promising because the cells survive transplantation into the mature rat brain and rescue the ability of the axotomized septal neurons to produce acethylcholine, which is lost after axotomy with no transplant [37•]. Fibroblastic 3T3 cells and endocrine RIN cell lines modified by the introduction of TH also survive transplantation into denervated striatum of rats with unilateral 6-OHDA lesions, where they continue to synthesize TH [38•]. The 3T3 cells secreted more dihydroxyphenylalanine (DOPA) than the RIN cells and more efficiently reversed apomorphine-induced turning behavior, but the use of either cell line as a therapy for human diseases is limited by the tumorigenicity of the cell lines. In a more promising approach designed to avoid both graft rejection and tumor formation, skin fibroblast cultures from inbred hosts were transfected with TH complimentary DNA and transplanted into the denervated striatum of rats with unilateral 6-OHDA lesions [39••]. The cells survived without forming tumors, synthesized TH and partially restored motor deficits as determined by the apomorphine induced rotation assay.

Conclusion

Experiments using transplants of embryonic CNS tissue continue to provide insights into the mechanisms of axon growth and recovery from injury. Promising new approaches are being developed in disease models which may provide the experimental background for using transplants as an effective therapy in humans.

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Intraspinal Transplants

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Transplants of embryonic central nervous system tissue have long been used to study axon growth during development and regeneration, and more recently to promote recovery in models of human diseases. Transplants of embryonic substantia nigra correct some of the deficits found in experimental Parkinson's disease, for example, by mechanisms that are thought to include release of neurotransmitter and reinnervation of host targets, as well as by stimulating growth of host axons. Similar mechanisms appear to allow intraspinal transplants of embryonic brainstem to reverse locomotor and autonomic deficits due to experimental spinal cord injuries. Embryonic spinal cord transplants offer an additional strategy for correcting the deficits of spinal cord injury because, by replacing damaged populations of neurons, they may mediate the restoration of connections between host neurons. We have found that spinal cord transplants permit regrowth of adult host axons resulting in reconstitution of synaptic complexes within the transplant that in many respects resemble normal synapses. Transplants of fetal spinal cord may also contribute to behavioral recovery by rescuing axotomized host neurons that otherwise would have died. Electrophysiological and behavioral investigations of functional recovery after intraspinal transplantation are preliminary, and the role of transplants in the treatment of human spinal cord injury is uncertain. Transplants are contributing to our understanding of the mechanisms of recovery, however, and are likely to play a role in the development of rational treatments.

Tessler A. Intraspinal transplants. Ann Neurol 1991;29:115–123

Transplants of embryonic central nervous system tissue have served for nearly a century in experiments designed to clarify the mechanisms that contribute to axon outgrowth and regeneration (reviewed in [1]). Throughout the past 20 years, transplants have been used in attempts to produce physiological or behavioral improvement in laboratory models of human diseases and to study the mechanisms that explain recovery of function (reviewed in [2–4]). Transplants remained primarily of theoretical interest for clinicians, however, until reports that autografts of adrenal medulla into the caudate nucleus produced modest [5] or dramatic [6] improvement in the motor behavior of patients with Parkinson's disease. More than 250 patients with Parkinson's disease have received adrenal medulla autografts [7], and additional patients have received transplants of human fetal substantia nigra [8–10]. The benefits of these procedures continue to be debated [11–17], and the mechanisms by which transplants elicit their effects continue to be investigated.

Transplants have also been reported to produce improvement in experimental models of Huntington's disease [2], Alzheimer's disease [18], and spinal cord injury [19–25]. Intraspinal transplantation would seem to be far from clinical application because electrophysiological and behavioral investigations of functional recovery are still preliminary. Regions of embryonic brain [26–35] as well as whole pieces or suspensions prepared from embryonic spinal cord survive transplantation into the spinal cord of adult and newborn host rats [36–38]. Intraspinal transplantations have also been successful in cat [21, 39, 40] and monkey. The extent to which connections form between transplant and host is beginning to be studied with morphological techniques.

In the present review, the strategies that have been proposed for using intraspinal transplants to reverse the deficits due to spinal cord damage will be considered, and the progress that has been made will be outlined. Because the effects of transplants have been most thoroughly studied in the basal ganglia in relation to Parkinson's disease, I first consider the mechanisms by which transplants may reverse the behavioral deficits in experimental models of this disorder.

Transplants for Experimental Parkinson's Disease

Transplantation strategies for the treatment of Parkinson's disease were built on a foundation of reproducible and quantifiable experimental models (reviewed in [41]). The first of these was a rodent model in which the substantia nigra corpus striatum projection was destroyed by the unilateral or bilateral stereotactic injection of the dopaminergic neurotoxin 6-hydroxydopa-

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mine (6-OHDA). When fetal substantia nigra was placed into the lateral ventricle adjacent to the corpus striatum that had been denervated by 6-OHDA, transplanted neurons survived and formed a dense dopaminergic innervation within the grafts, but few processes extended into the host corpus striatum [42]. In spite of the limited reinnervation, the transplants improved some aspects of motor behavior. This result suggested that reinnervation was not necessary for recovery and that release of dopamine into the cerebrospinal fluid and in the vicinity of the target neurons sufficed. If fetal substantia nigra is transplanted into the parenchyma of the corpus striatum denervated by 6-OHDA injection rather than into the ventricle, then the transplants partially restore the damaged neural circuits and additional behaviors recover [43–47]. At least some of the recovery is related to reinnervation of the host striatum (reviewed in [41, 48]). Other behavioral deficits induced by unilateral dopamine deafferentation, however, remain uncorrected by the transplants. Incomplete behavioral recovery has been attributed to incomplete restoration of the damaged regulatory circuitry [47].

Another mechanism by which transplants may compensate for behavioral deficits has been demonstrated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of experimental Parkinson's disease [49, 50]. When adrenal medullary cells are transplanted into the striatum of mice intoxicated with MPTP, the mice recover in spite of a very limited survival of grafted cells [51]. The expression of tyrosine hydroxylase, a marker for dopaminergic neurons, increases within the caudate ipsilateral to the site of MPTP injection, and derives from axons of the host dopaminergic projection rather than from the few surviving transplanted neurons [51]. Increased enzyme expression indicates regeneration of damaged host axons or collateral sprouting of undamaged host axons and suggests that the transplant or the damage associated with the transplant procedure [52] exerts a trophic effect on the host neurons that contributes to behavioral recovery but is independent of graft survival.

In summary, this experience with experimental Parkinson's disease indicates that some types of motor function can be improved by transplants that deliver neurotransmitters, trophic factors, or both, to the striatum even if the transplants fail to reconstitute the damaged neural circuits in all their detail. Because restitution of the damaged circuits is incomplete, however, the motor deficits are only partially compensated, and behavioral recovery is also incomplete.

Intraspinal Transplants

As elsewhere in the central nervous system, intraspinal grafts of peripheral nerve segments support axon growth [53, 54]. These experiments have contributed to the current view that many neurons once thought incapable of growth can elongate if provided with a suitable glial environment (reviewed in [55]). Although central nervous system axons can grow within sciatic nerve grafts for distances that exceed their normal length [56] and establish synaptic connections with neurons in the host parenchyma [57, 58], growth into the central nervous system beyond the graft is limited to 1 to 2 mm [55]. Two other strategies for using transplants to treat experimental spinal cord injury have therefore received considerable attention.

Intraspinal Transplants of Supraspinal Neurons

One strategy is based on the idea that transplantation of brainstem monoaminergic neurons important for regulating the activity of spinal neurons should mediate recovery even without the reconstitution of damaged neural circuits ([30] and reviewed in [35]). Both locomotor and autonomic functions might benefit. It is known, for example, that the intravenous administration of dopamine or the alpha$_2$-adrenergic agonist clonidine to cats with acute spinal cord transection can elicit stepping movements that permit walking on a treadmill [59]. Untreated, such cats are completely paraplegic after transection. These agents are thought to act directly on lumbar spinal cord segments to activate the circuitry for locomotion that is intrinsic to the spinal cord [60] but nonfunctional in the acute stage after transection. It is also known that serotoninergic neurons whose perikarya are in the brainstem raphe nuclei are important in the supraspinal control of spinal reflexes that mediate penile erection and ejaculation, and that administration of a serotonin receptor agonist to spinalized rats can induce ejaculation [61].

One approach to the treatment of behavioral deficits after experimental spinal cord injury has been to transplant supraspinal monoaminergic neurons into the caudal portion of the spinal cord isolated by transection. Both catecholaminergic neurons important for locomotion and serotoninergic neurons important for autonomic function have been used [62]. Embryonic noradrenergic neurons taken from locus ceruleus [31–35] and serotoninergic neurons taken from the mesencephalic or medullary raphe regions [23, 24, 63, 64] extend processes up to 1 to 2 cm in length into the host spinal cord. These transplants restore levels of neurotransmitters that have been depleted by the use of neurotoxins [34] or by spinal cord transection [23, 24]. Axons of transplanted serotoninergic neurons innervate the regions of spinal cord that receive 5-hydroxytryptamine (5-HT) innervation normally, including laminae I and II of the dorsal horn, the motoneuron area in lamina IX of the ventral horn, and the intermediolateral column [23, 24]. The transplanted serotoninergic axons establish axodendritic synapses on host motoneurons and axodendritic and axosomatic

synapses on neurons of the host intermediolateral column that resemble those formed by serotoninergic neurons in normal spinal cord [23, 24]. Additionally, reflex ejaculation that is abolished in rats with spinal cord transection recovers in rats that receive serotoninergic transplants of embryonic raphe neurons, but only rarely in rats that undergo transection alone or transection with transplantation of nonserotoninergic neurons [23, 24]. This result suggests that behavioral recovery is related to the recovery of serotoninergic innervation.

Noradrenergic axons originating in locus ceruleus transplanted into spinal cord also extend into the intermediate and ventral regions of the host caudal gray matter [34] to which locus ceruleus axons project in normal spinal cord. These axons are thought to contribute to the recovery of hindlimb flexion reflexes in rats whose spinal cord catecholamines have been depleted by an intracisternal injection of 6-OHDA [20] and to the recovery of reflex stepping activity in rats with spinal cord transections [25]. Whether transplanted noradrenergic axons establish synapses with host neurons has not been studied, and therefore, the anatomical basis for the locomotor recovery mediated by the transplants is uncertain. Like intraparenchymal transplants of substantia nigra in models of Parkinson's disease, these embryonic transplants of brainstem neurons appear to reinnervate a portion of their normal targets and contribute to behavioral recovery by releasing neurotransmitters onto their normal targets or in their vicinity. The release of these neurotransmitters would then mimic the modulatory activity of descending noradrenergic systems in normal spinal cord and suffice to activate the intrinsic spinal pattern generators for locomotion [34, 60]. As in the corpus striatum deafferented by 6-OHDA, the transplants are successful although they have been placed into abnormal locations and can only incompletely restore the interrupted neural circuits.

Transplants of Fetal Spinal Cord

The recovery of other functions lost after spinal cord injury such as discriminatory sensation or fine motor control is likely to require more faithful reconstruction of normal circuitry than is possible by using transplants of supraspinal neurons. A second strategy has therefore been to use transplants of fetal spinal cord in an attempt to replace damaged populations of neurons and encourage the restoration of connections between neurons [36, 65]. According to this rationale, the transplant would act as a bridge across damaged tissue either by allowing injured axons to grow directly into intact spinal segments or by permitting the establishment of relays within the graft (Fig 1). Another mechanism provided by transplants might be the rescue of axotomized neurons in the spinal cord. Although the spinal cord has been considered a technically challenging site in

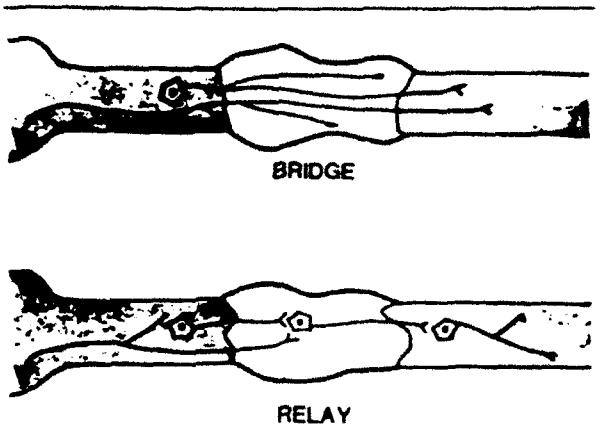
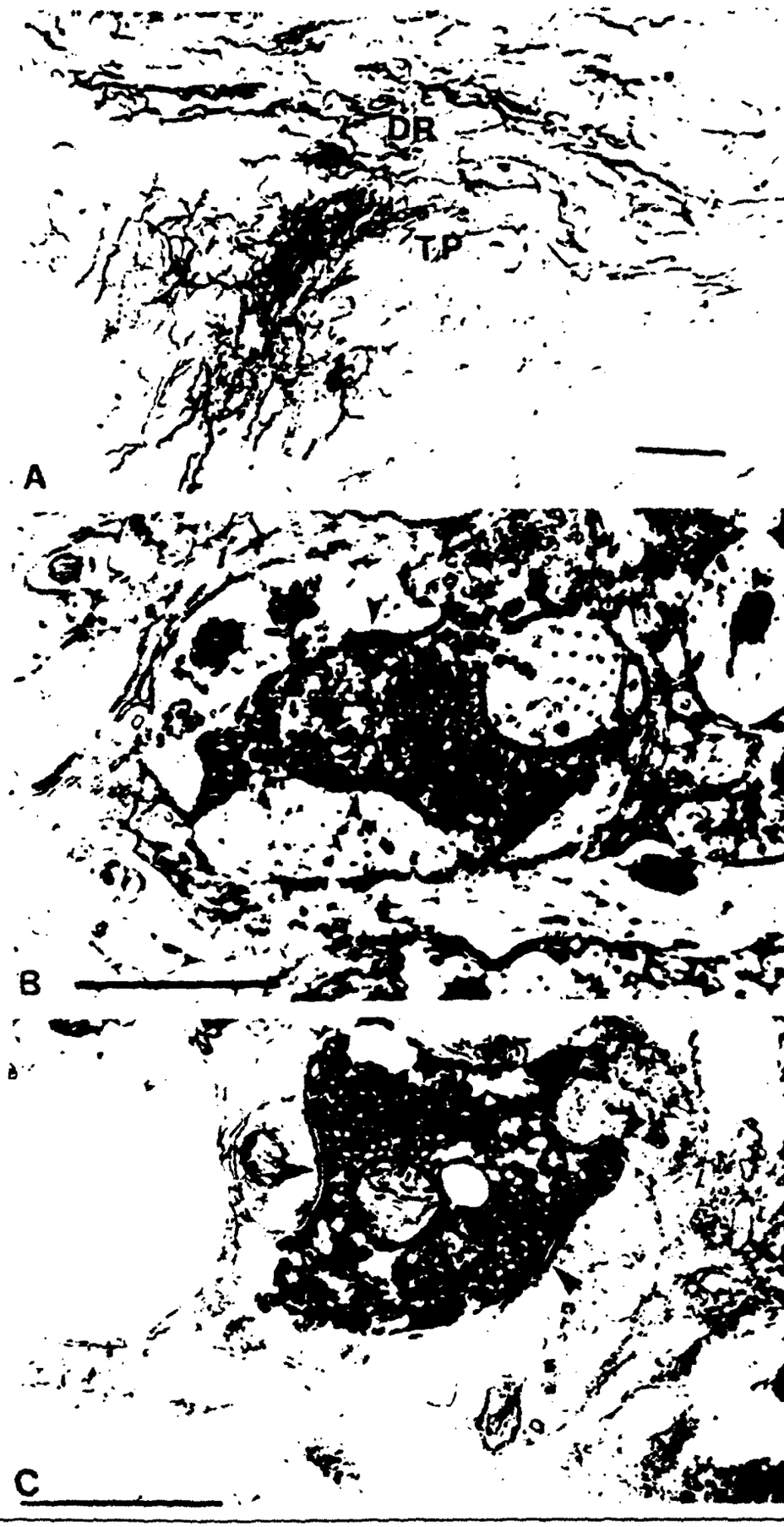


Fig 1. A diagram illustrating two ways in which a transplant (shaded area) at a region of spinal cord injury might promote functional recovery. Host brain and spinal cord rostral to the transplant are to the left and host caudal spinal cord is to the right. (Bridge) Injured axons originating in host neurons rostral to the transplant grow directly through the transplant into caudal host spinal cord. (Relay) Injured axons originating in host neurons rostral to the transplant grow into the transplant and synapse with donor neurons whose axons grow into caudal host spinal cord. (Courtesy of Dr Barbara Bregman.)

which to obtain survival of embryonic spinal cord transplants, with present methods 80 to 90% of transplants survive grafting into the acutely injured adult or newborn spinal cord [28, 36] or into the chronically injured adult spinal cord [37]. Transplant survival is now possible also in the site of complete spinal cord transection [21, 66, 67].

Several morphological features of the transplants encourage the expectation that they might replace damaged populations of neurons and with them their normal connections. First, although transplants lack the characteristic butterfly appearance of normal spinal cord gray matter, they do contain differentiated areas that resemble substantia gelatinosa based on several morphological criteria [68]. Second, areas of apposition between transplant and host develop, particularly between transplant and host spinal cord gray matter, in which the interface is free of astrocytic scarring and processes pass between transplant and host [36]. It has, in fact, been suggested both that embryonic transplants reduce the astrocytic scarring that accompanies acute spinal cord injury and may impede regeneration [69, 70] and that they can reduce an already established astrocytic scar [37].

One additional way in which transplants of embryonic spinal cord may contribute to recovery of behavioral function was first described in newborn rat hosts, where transplants rescue axotomized host rubrospinal neurons that otherwise would have died [71]. Permanent rescue is target specific because only transplants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of fetal spinal cord support survival at long survival periods; both target and nontarget transplants support short-term survival [72]. Axotomized neurons of Clarke's nucleus can also be rescued by embryonic transplants in newborns, and a rescue of similar magnitude has now been reported when the same population of neurons is axotomized in adult animals [73].

In adults, development of projections from host into transplant and from transplant into host is modest. Host axons that have grown into the transplants do not traverse their full extent and do not reenter host spinal cord [36]. Morphological tracing methods have shown that donor neurons project for distances of 5 to 7 mm into host spinal cord and that host neurons in adjacent spinal segments project from 3 to 5 mm into the transplants [36]. Additionally, serotoninergic axons from brainstem raphe nuclei [36], axons from corticospinal neurons [74, 75], and primary afferent axons originating in host dorsal root ganglion neurons [67, 76] regenerate into transplants although they are unable to regenerate into adult spinal cord in the absence of a transplant (Fig 2). The terminals of regenerated dorsal root axons establish synapses within transplants of embryonic spinal cord, and these synapses resemble those formed by primary afferent axons in the dorsal horn of normal animals [38] (Fig 3). Differences were also observed, however, particularly an increased percentage of axo-axonic synapses suggesting that regenerated dorsal root axon terminals had formed connections with each other. Nevertheless, the establishment of synapses that are morphologically normal by an identified set of host neurons afferent to the transplants encourages the idea that neurons joined by synapses within the transplants may enable the transplants to act as relays across regions of damaged spinal cord. Donor neurons within transplants have in fact been shown to send axons into the host sciatic nerve [76] as well as into peripheral nerve grafts introduced into the transplants [77, 78]. Whether these axons reach and innervate host muscles is unknown. It is, however, at least possible that transplants of embryonic spinal cord not only can support or enhance the regeneration of adult axons otherwise unable to grow but also that they can contribute to the reestablishment of a segmental motor reflex arc.

In newborn rat hosts, spinal cord transplants act as bridges that encourage or permit the growth of supraspinal neurons into segments of spinal cord well below the level of injury. Serotoninergic axons originating in neurons of the brainstem raphe [79] and axons of corticospinal neurons [75] grow across transplants placed in the lesioned thoracic spinal cord of newborn rats and terminate in their normal target areas as far caudal as the lower lumbar segments of host spinal cord. Because these systems of neurons are among the descending pathways that contribute to the control of locomotion, the possibility has been tested that transplants alter the development or enhance the recovery of motor function after spinal cord injury in newborn rats with partial spinal cord lesions [22]. When studied with a battery of tests of locomotor function, newborn rats with transplants of embryonic spinal cord performed better than littermates with thoracic spinal cord injuries alone. For example, when tested 8 to 12 weeks postoperatively, rats with transplants crossed a mesh runway more quickly and made fewer errors in foot placement than the lesion-only group of rats. They also recovered more quickly from their errors. These results are consistent with the idea that the axons that grow into host spinal cord caudal to the thoracic spinal cord injury contribute to the improved performance. Because the rats in this study sustained an incomplete injury rather than complete spinal cord transection, however, other explanations cannot be excluded. One possibility is that the transplants altered the response to injury of the remaining spinal cord and slowed or decreased degeneration, thus providing a greater amount of healthy tissue through which developing or regenerating axons might grow. Corticospinal axons are known to grow through undamaged areas of spinal cord adjacent to an incomplete spinal cord injury in newborns [80–82], and transplants of cultured dorsal root ganglia neurons and Schwann cells have been shown to enhance this growth [83]. Although corticospinal axons grow more robustly through transplants than through the remaining portions of spinal cord [75], supraspinal axons that have grown through host spinal cord as well as those that have grown through the transplant may contribute to the recovery.

Locomotor function is also being studied in cats that have received embryonic spinal cord transplants into the site of spinal cord transection on the day after birth. These preliminary studies on cats with transections complement those performed in rats with hemisections because a more detailed analysis of locomotor function is possible in cats. Additionally, the anatomical pathways that explain performance on different tests of mo-

Fig 2. Light (A) and electron (B,C) micrographs of embryonic spinal cord transplants. (A) Sagittal section 1 month after transplantation stained with an immunocytochemical method for demonstrating calcitonin gene-related peptide (CGRP). CGRP originates in many dorsal root ganglion neurons and serves as a marker for host dorsal roots (DR) that have regenerated into the transplant (TP) and arborized there (29, 38, 86). Bar = 100 μm. (B) CGRP-labeled complex terminal in a transplant makes contacts (arrowheads) *with two dendritic profiles. Bar = 1 μm. (C) Host dorsal roots labeled with horseradish peroxidase (HRP). Complex terminal that has been filled with HRP makes synaptic contacts* (arrowheads) *in a transplant with three dendritic profiles. Bar = 1 μm.*

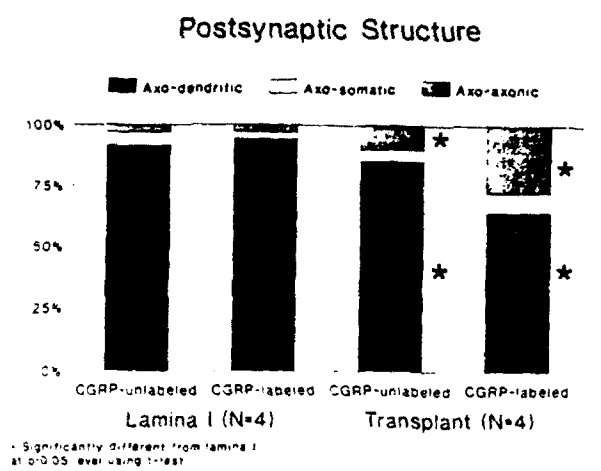

Fig 3. Comparison of synaptic contacts found in embryonic spinal cord transplants and lamina I of normal spinal cord. Calcitonin gene-related peptide (CGRP)-labeled synaptic terminals are derived from dorsal roots; the origin of unlabeled synaptic terminals is undetermined. Compared with lamina I, both CGRP-labeled and CGRP-unlabeled terminals in transplants make significantly greater numbers of axo-axonic synapses and significantly smaller numbers of axo-dendritic synapses. Axo-dendritic synapses predominate in transplants, however, as in lamina I. For details of the stereological analysis see (38).

tor function are better defined in cats than rats. Previous studies in normal [84] and cats with spinal cord transection [85, 86, 87] have demonstrated three types of locomotion that are available for analysis and that are controlled by different classes of spinal pathways [88]. For example, after resolution of the period of reflex depression known as spinal shock, hindlimb locomotion in response to a moving treadmill (bipedal reflex locomotion) returns in adult cats with spinal cord transection (spinal cats) because this type of locomotion requires only that the spinal pattern generators for each hindlimb and the connections between them remain intact [86]. Reflex locomotion on a treadmill that requires coordination between the forelimbs and hindlimbs (quadrupedal reflex locomotion) does not recover after thoracic transection because it depends on propriospinal connections between forelimb and hindlimb pattern generators in the cervical and lumbar spinal cord, and these are interrupted by the transection. Conditioned (voluntary) overground locomotion for a food reward will also not recover after transection either in adult or newborn cats because it depends not only on intact segmental and intersegmental connections but also on the presence of pathways that originate in the brain. In cats with thoracic spinal cord transections that have received a transplant on the day after birth, the presence of quadrupedal reflex locomotion will, therefore, suggest that the transplant has encouraged the growth of propriospinal connections across the site of transection, and the presence of conditioned overground locomotion will suggest the growth of axons with cell bodies in the brain.

Locomotor function has been examined in two cats that received transplants of E-26 spinal cord into T-12 transections on the day after birth [21]. These cats were compared with normal cats [89] and with a previously reported group of cats with transections as newborns but which did not receive transplants (spinal cats) [86]. By 3 weeks of age, the transected cats with transplants begin to differ from the spinal cats and the differences are maintained throughout the period of study. All groups of cats develop bipedal stepping, but unlike spinal cats, those with transplants also develop both full weight-supported quadrupedal stepping on a treadmill and overground locomotion. During overground locomotion, cats with transplants develop patterns of limb movement that suggest coordination between hindlimbs and forelimbs. The coordination is only sometimes similar to that seen in normal adult animals and overground locomotion is abnormal. For example, the postural stability of the hindquarters is impaired, the step cycle is prolonged, and the normal 1:1 pairing of forelimb and hindlimb step cycles is inconsistent. When the cats were studied histologically 2.5 and 8 months after transplantation, transplants filled the entire lesion cavity and were in continuity with host spinal cord in some places. Both transplants developed areas of gray matter and ependymal elements, and contained healthy appearing neurons and glial cells. These preliminary results indicate that transplants enhance the development or recovery of locomotor function in newborns with spinal cord transection and suggest that this effect is mediated by the axons of propriospinal or supraspinal neurons, or both, that enter the transplants. Additional experiments using anatomical tracing methods are necessary to establish this and to determine whether these axons traverse the transplant or form multisynaptic relays within the transplant. If the locomotor behavior in fact depends on supraspinal input, then transplants into newborn spinal cord will have assisted in the development or recovery of complex behavior that requires the reconstitution of supraspinal input and control of these supraspinal neurons by the host. Like the transplants for experimental Parkinson's disease, these transplants restore some of the lost functions but not the complete array.

Greater recovery may be obtained by combining transplants with additional agents that encourage axon growth. When exposed to the joint influences of a fetal spinal cord transplant and a prosthesis containing nerve growth factor (NGF), for example, cut dorsal root axons grow into and past the transplant into the ventral horn of host spinal cord [90]. Without added NGF, dorsal roots grow into the grafts but not through them

into host spinal cord [76]. NGF is the best studied neurotrophic factor and is known to elicit axon outgrowth in vitro [91]. In addition to growth factors, other agents are becoming available that, together with a transplant, might promote growth past an area of damage and into undamaged spinal cord. Increasing experimental evidence now emphasizes the importance of inhibitory influences in directing and limiting axon growth [92–95], and the molecules responsible are beginning to be identified. Caroni and Schwab [96, 97], for example, have demonstrated that peptides present in central nervous system myelin inhibit axon extension [96] and that this inhibitory activity can be blocked with a monoclonal antibody against the peptides [97]. Application of these antibodies to young rats whose corticospinal axons have been transected in the thoracic spinal cord is associated with sprouting of these axons at the lesion site and limited growth into the caudal spinal cord [98]. Scarring at the lesion site may obstruct the growth of many of these sprouted axons [70, 99]. Because transplants are reported to reduce gliosis and support axon extension across an area of injury, growth and associated recovery might be greater if, in addition to reducing inhibitory influences, the lesion site were bridged with a transplant.

Conclusion

Intraspinal transplants have clarified the conditions necessary for axon growth and regeneration, and have provided strategies that can contribute to behavioral recovery in experimental models of disease. Many issues remain to be resolved in the laboratory before the therapeutic potential of transplants can be evaluated. That injured central nervous system neurons can survive and grow in an appropriate environment, however, increases the likelihood that rational therapies to promote recovery will be forthcoming and makes more urgent the basic studies on which the development of these therapies will depend. Although it is not yet clear whether transplants will be part of the treatment of human spinal cord disease, they will at least have a role to play in developing treatments.

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CHAPTER 14

DOES NEURAL TRANSPLANTATION AID THE RECOVERY OF CNS FUNCTION?

Alan R. Tessler, M.D.

When Sir Peter Medawar was asked about the application of his work on experimental transplantation he responded, "nothing." Experimentalists are no longer so self-assured about gaining knowledge for knowledge's sake, nor are they likely to be as wrong about the application of their work as Medawar was.

Santiago Ramón y Cajal first showed the feasibility of nervous system transplantation and its regenerative potential. Tessler provides evidence for optimism in regard to transplantation for Parkinson's disease and spinal cord injury.

The ultimate limits to transplant treatment of human disease may well prove to be not scientific but ethical, as the recent raging debate on fetal transplantation in the British House of Lords suggests.

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Transplants of embryonic nervous system tissue have been used since the end of the nineteenth century as an experimental strategy for studying the mechanisms by which axons develop and regenerate.[19] Although transplants have been the subject of a steadily increasing number of publications by basic scientists for the past 25 years, their interest for clinicians increased dramatically in the mid-1980s with reports from Sweden and Mexico that autografts of adrenal medulla produced modest[1] or major[35] improvement in the motor performance of patients with Parkinson's disease. Over 100 patients in the United States and additional patients abroad have undergone this operation, and the benefits of the procedure, as well as the mechanisms by which transplants exert their effects, remain a subject of considerable controversy and ongoing investigation. Transplants hold additional interest for neurologists and neurosurgeons because they have been reported to produce improvement in experimental models of human diseases that include not only Parkinson's but also Huntington's disease,[5] Alzheimer's disease,[12] and spinal cord injury.[46] Their potential clinical application is therefore extremely broad.

The mechanisms by which transplants promote behavioral recovery are likely to be complex and may differ depending on the identity of the tissue that is transplanted, the condition for which the tissue is transplanted, and the site into which the graft is placed.[18] The goal of transplantation, however, is to promote behavioral recovery by restoring or replacing as much of the damaged neuronal circuitry as is necessary to be functionally effective. The extent to which

the circuitry will have to be restored to normal depends on the system that has been damaged. In some cases specific connections between neurons may need to be replaced, and the transplanted neurons may have to be thoroughly integrated into surviving neuronal circuitry. Such a complete restoration of circuitry might be necessary, for example, to restore the fine motor functions mediated by corticospinal neurons or the discriminatory sensations mediated by primary afferent neurons. In other cases it might suffice for transplants to act as pumps of neurotransmitter that would ensure a constant supply of neurotransmitter within the cerebrospinal fluid or close to the targets of damaged neurons. Restoring the functions mediated by still other systems of neurons might require intermediate degrees of specificity of connections between host neurons afferent to transplants and transplant neurons efferent to host target neurons. The present brief review will consider one experimental model in which transplants have been used to correct deficits that do not require complete restoration of specific connections between donor and host tissues and one model in which the restoration of function is likely to require the establishment of specific circuits. As an example of the restoration of function primarily through the release of neurotransmitter, we will consider the experimental background that led to the use of transplants for the treatment of Parkinson's disease. As an example of restoration of function that is likely to require the replacement of specific connections, we will consider experiments that may lead to a similar treatment of spinal cord injury.

TRANSPLANTS FOR PARKINSON'S DISEASE

Appropriateness of Transplant Therapy

Features of Parkinson's Disease

Several features of Parkinson's disease make it a promising candidate for treatment by the transplantation of neural tissue. First, the disordered motor function is largely accounted for by the degeneration of a single system of dopaminergic neurons whose cell bodies are in the substantia nigra and whose axons terminate in the ipsilateral caudate and putamen.[25] Transplanted tissue would therefore have only to replace the spatially restricted projections of a discrete population of neurons. Second, levels of striatal dopamine must be reduced by at least 80% before the disease becomes symptomatic.[25] Transplanted tissue might therefore improve function even if levels of dopamine remained far below normal. Third, the dopaminergic neurons whose degeneration is responsible for the motor dysfunction of Parkinson's disease are thought to be "permissive" neurons that modulate the level of activity of neurons in the striatum by providing nonspecific information related to arousal.[10,54] This background level of activity is necessary for neuronal interactions within the striatum to occur, but it is set not by precise point-to-point connections with postsynaptic targets but by the release of transmitter in proximity to these targets.[10] Transplants would therefore not necessarily have to restore the synaptic connections found in the normal striatum but might function usefully as reservoirs for the release of neurotransmitter in the vicinity of their targets in the striatum. Fourth, treatment with levodopa or carbidopa often relieves, at least temporarily, the motor impairments of Parkinson's disease in spite of the continuing degeneration of substantia nigra neurons. Because the exogenous replacement of neurotransmitter is effective, transplants might function even in the absence of either a normal complement of connections between transplant and target neurons or an extensive network of afferent neurons from the host.

Availability of Experimental Model

Information available from studies performed in several laboratories encouraged attempts to use transplants in the treatment of experimental parkinsonism. It was known, for example, that transplants of embryonic substantia nigra survived grafting to the anterior chamber of the eye and that neurites would grow out from these transplants and establish projections within

pieces of embryonic corpus striatum that has been cotransplanted along with the substantia nigra.[41] A quantifiable and particularly useful laboratory model for Parkinson's disease also existed in which one substantia nigra–corpus striatum projection was destroyed in rats by a unilateral sterotaxic injection of the neurotoxin 6-hydroxydopamine (6-OHDA).[10,41] Destruction of one projection produced supersensitivity of the denervated dopaminergic receptors and consequently a syndrome in which rats turned away from the lesion following the systemic injection of apomorphine. This is a dopamine receptor agonist that stimulates denervated receptors to a greater extent than those that are intact. The systemic administration of amphetamine, which causes the release of dopamine from intact terminals, caused the animal to rotate toward the side of the injection.[10,41] Because the circling behavior of these rats could be quantified, it was possible to transplant embryonic substantia nigra adjacent to the corpus striatum of rats that had received a unilateral 6-OHDA lesion and to expect (1) that the grafts would survive and establish projections into the corpus striatum and (2) that the behavioral consequences of the transplantation could be studied quantitatively.

Transplants in Experimental Parkinsonism

Fetal Substantia Nigra Transplants in 6-OHDA Rodent Model

In one of the early demonstrations that transplants could reverse experimental parkinsonism, fetal substantia nigra was placed into the lateral ventricle adjacent to the caudate nucleus, which had been denervated by an injection of 6-OHDA.[44] The grafted neurons survived and established a dense dopaminergic innervation within the transplant, but very little outgrowth into host parenchyma occurred. Nevertheless, rats that had received a transplant of fetal substantia nigra, but not those that had received a control transplant of sciatic nerve, demonstrated behavioral improvement on the apomorphine-induced rotation test. This result suggested that the test transplants had reduced the supersensitivity of denervated dopaminergic receptors and that the mechanism did not require reinnervation of the denervated receptor sites. Release of dopamine into the cerebrospinal fluid and elsewhere in the vicinity of the denervated striatum appeared to suffice. When, however, solid pieces of fetal substantia nigra are grafted onto the surface of the neostriatum denervated by 6-OHDA injections,[27] or nigral cell suspensions are injected into the striatum,[4] large numbers of dopaminergic axons grow for distances of up to 2 mm into the adjacent denervated host striatum. Dopamine is released from transplanted neurons,[48] and levels of dopamine[17] as well as the density of postsynaptic dopamine receptor binding sites[15] in the striatum denervated by 6-OHDA return toward normal. Both behavioral and morphologic observations support the idea that the recovery is mediated at least in part by reinnervation of host striatum as well as by local release of neurotransmitter. For example, dopaminergic axons originating in the transplants form synapses in host neuropil.[36] In addition, outgrowth appears to be correlated with behavioral improvement, since asymmetries in rotation induced by 6-OHDA injection are corrected by transplants that send projections into the dorsal striatum but not into the lateral neostriatum, whereas the sensorimotor "neglect" of the side contralateral to a unilateral injection of 6-OHDA is counteracted by transplants that send axons into the ventrolateral portions of the neostriatum.[41]

Fetal Substantia Nigra Transplants in MHTP Primate Model

The administration systemically of the meperidine analog MPTP produces in monkeys,[9] as well as in humans,[30] a selective degeneration of nigrostriatal dopamine neurons, which results in impairments in motor behavior similar to those of Parkinson's disease. This neurotoxin therefore provides an experimental model in monkeys in which the morphologic, biochemical, and behavioral consequences of transplants can be studied. When solid grafts of fetal substantia nigra are transplanted into the striatum of adult African green monkeys intoxicated with MPTP, large numbers of

grafted tyrosine hydroxylase–containing, presumably dopaminergic neurons extend processes into the denervated striatum, and levels of the dopamine metabolite homovanillic acid in the cerebrospinal fluid, which are decreased by MPTP, return toward normal.[51] The impairments in motor performance also recover in the early days after transplantation, and improvement is found months later, although there may be an intervening period of relapse. This recovery is likely to be due to reinnervation, since a control monkey that received transplants of fetal substantia nigra into cingulate cortex, fetal hypothalamic dopaminergic neurons into one striatum, and noradrenergic neurons from locus ceruleus and subceruleus into the other striatum showed only transient improvement in spite of the survival of transplanted neurons.[51] Because the behavioral deficits are corrected by embryonic substantia nigra transplants that have been placed adjacent to the target rather than in the normal position of dopaminergic neurons in the midbrain, host afferent input to the transplant must differ from the pathways that modulate substantia nigra function in the normal brain. The restoration of motor function that is mediated by transplants in the 6-OHDA and MPTP models of Parkinson's disease therefore does not require the restoration of normal neuronal circuitry.

Adrenal Medulla Transplants in Rodent and Primate Models

To avoid the complicated practical and legal problems that would be raised by transplants of fetal tissue in humans, the behavioral effects of other sources of dopaminergic neurons have been investigated. When separated from the adrenal cortex and cultured in an environment that contains nerve growth factor (NGF), chromaffin cells of the adrenal medulla have been shown to extend neurites and synthesize catecholamines including dopamine.[42] Intraventricular transplants of adrenal chromaffin tissue reverse apomorphine-induced turning behavior to about the same extent as fetal substantia nigra transplants in rats whose striatum has been unilaterally denervated by 6-OHDA; very few neurites are produced by these transplants.[16] Intrastriatal transplants of adrenal medulla also produce behavioral improvement in the 6-OHDA–treated rodent model, but, unlike transplants of embryonic substantia nigra, this is not due to reinnervation of the denervated striatum, since intraparenchymal transplants of adrenal medulla form very limited projections.[14,43] Diffusion of dopamine is also unlikely to account for recovery after adrenal medulla transplants, because neither cerebrospinal[2,55] nor striatal[2] levels of catecholamines are persistently elevated after intrastriatal transplantation.

An alternative explanation is that the transplant or the transplantation procedure itself induces dopaminergic neurons in the host striatum to regenerate, to form collateral axons, or to increase their synthesis of catecholamines[39] and that it is this compensatory response of dopaminergic host neurons that is crucial for the behavioral recovery. Transplantation of adrenal medullary cells into the striatum of mice intoxicated with MPTP, for example, is associated with increased density of host tyrosine hydroxylase–containing axons that occurs despite a very limited survival of grafted cells.[5] This result suggests that the transplant exerts a trophic effect on the host neurons that is independent of graft survival. Enhanced outgrowth of tyrosine hydroxylase–positive fibers is also observed near graft sites in the caudate of normal Cebus monkeys or of Cebus monkeys treated with MPTP,[21] although very few adrenal chromaffin cells survive transplantation.[11] However, tyrosine hydroxylase–immunoreactive fibers were also induced in a control monkey that had received an implant consisting only of a metal tissue carrier.[11] One implication of this experiment is, therefore, that the injury caused by the transplant procedure, rather than the tissue implanted, produces compensatory responses in the host that contribute to behavioral recovery.[11,29,38]

Transplants in Parkinson's Disease Patients

Whether or not transplants of adrenal medulla produce behavioral improvement in

the primate model for Parkinson's disease has received surprisingly little attention. In human beings with Parkinson's disease the results have been mixed. As reported from Sweden, four patients received transplants of solid pieces of their own adrenal medulla into either the caudate[1] or the putamen.[33] At best the procedures produced transient improvement shown by brief reversal of motor deficits and temporary decrease in the need for medication. More dramatic and sustained improvement was reported by Madrazo and colleagues[35] in a series of patients that now totals over 40. The two patients described in their initial report showed a marked reduction in rigidity, akinesia, and tremor that persisted for the duration of the follow-up periods of 3 and 10 months. The operation as performed in Mexico differed technically from that performed in Sweden because the procedure was performed as an open craniotomy, and the solid graft implanted into the caudate remained in contact with the cerebrospinal fluid of the lateral ventricle. Differences in surgical technique alone, however, are unlikely to have accounted for the differing outcomes, because other groups that have employed the same surgical procedure as was used by the Mexican surgeons have been unable to reproduce their results. A study of 19 patients that combined the results of three medical centers in the United States, for example, found only an increase in the periods in which patients responded to medication ("on" time) and a decrease in the severity of impairment during the periods when the medication was ineffective ("off" time).[20] Postoperative medical and behavioral complications were also frequent. In general, the results of the more than 100 operations performed in the United States have shown that the procedure produces "modest to slight benefits in only a proportion of patients"[52] and have prompted considerable skepticism about the success reported by Madrazo and co-workers.[32] The operation has failed when the transplant has not survived,[45] and when chromaffin cells have survived in the transplant but not synthesized dopamine,[22] suggesting that in the human with Parkinson's disease, graft survival and dopamine synthesis are necessary to improve motor performance and that compensatory changes in the host produced by the transplant or the transplantation procedure do not suffice.

If the capacity to reinnervate the striatum and to deliver dopamine close to the normal target neurons is a prerequisite for transplants to mediate locomotor recovery, then the available experimental evidence suggests that transplants of embryonic substantia nigra provide a more promising therapeutic possibility than adrenal medulla transplants. Two patients who received transplants of human fetal substantia nigra tissue have not, however, shown major functional improvement after a 6-month period of study.[34] Formidable technical problems need to be solved in the rodent and primate models of Parkinson's disease before the still more difficult technical challenges as well as complicated legal and ethical issues in humans are confronted.

TRANSPLANTS FOR SPINAL CORD INJURY

State of the Art

Transplantation is far from providing a practical approach to the treatment of patients with spinal cord disease or spinal cord injury. Areas of embryonic brain as well as whole pieces and cell suspensions prepared from embryonic spinal cord have been shown to survive transplantation into the spinal cord of newborn and adult host rats, and the possibility that connections form between neurons in the host and neurons in the transplant has begun to be evaluated using a variety of morphologic methods. The electrophysiologic and behavioral function of these transplants is at an earlier stage of study, and very little work has been done yet in the cat or primate.

Transplant Strategies

Transplants of Fetal Supraspinal Neurons

One implication of the transplant studies in the experimental models of Parkinson's disease is that some types of behavior can

be improved by transplants that deliver neurotransmitters and/or neurotrophic substances to the vicinity of their targets, even if the transplants cannot reconstitute the damaged neuronal circuits in detail. One strategy for using transplants to correct experimental spinal cord injury has followed similar reasoning: that the appropriate neurons placed in proximity to their targets can restore some behaviors abolished by spinal cord injury even without the complete restoration of damaged circuits.[40] It is known, for example, that the intravenous administration of dopamine or the alpha$_2$-adrenergic agonist clonidine can elicit stepping movements in cats with spinal cord transection that allow them to walk on a treadmill.[13] This action is thought to be due to the activation of circuitry for locomotion that is intrinsic to the spinal cord. It is also known that serotonergic neurons are important in the supraspinal control of the spinal reflexes that mediate penile erection and ejaculation and that administration of a serotonin receptor agonist to rats with spinal cord transection can induce ejaculation.[37] Therefore, one approach has been to transplant supraspinal monoaminergic neurons into the distal portion of spinal cord isolated by a lesion. Both catecholaminergic neurons known to be important for locomotor behavior and serotonergic neurons that mediate autonomic function have been used. Embryonic noradrenergic neurons taken from locus ceruleus and serotonergic neurons taken from the rhombencephalic raphe region extend processes up to 2 cm in length into host spinal cord and restore levels of neurotransmitters that have been depleted experimentally by the use of neurotoxins[40] or by spinal cord section.[46] Axons of transplanted serotonergic neurons grow into the regions of spinal cord that receive 5-HT innervation normally and establish morphologically normal synaptic terminals with motoneurons in the ventral horn and with neurons in the intermediolateral column.[46] In addition, reflex ejaculation, which is abolished in rats with spinal cord transection, recovers in rats that receive transplants of embryonic raphe,[46] suggesting that behavioral recovery is related to the recovery of serotonergic innervation.

Transplanted noradrenergic neurons originating in the locus ceruleus also extend axons into the regions of spinal cord to which locus ceruleus axons project in normal spinal cord and appear to contribute to the recovery of reflex stepping activity.[57] The question of whether transplanted noradrenergic axons establish synapses with their target neurons has not been studied, and therefore the anatomic basis for the recovery mediated by noradrenergic neurons remains to be determined. Like transplants of embryonic substantia nigra in experimental Parkinson's disease, these embryonic transplants of brainstem nuclei appear to contribute to behavioral recovery by extending axons into host neuropil, where they function by releasing neurotransmitter or neurotrophic substances close to their normal targets. As in the parkinsonism models, the transplants are successful even though they have not been placed in their normal location and the neuronal circuits in which they participate normally have not been restored.

Transplants of Fetal Spinal Cord

Because the recovery of other behaviors abolished by spinal cord injury may require more faithful reconstruction of damaged circuits, a second strategy has been to use transplants of embryonic spinal cord in an effort to replace populations of damaged neurons and restore neuronal connections.[47] The transplant might then function as a bridge across injured tissue, either by permitting the direct growth of lesioned axons into intact spinal segments or by permitting the formation of relays within the graft. Embryonic spinal cord transplanted into a cavity in host spinal cord survives and becomes integrated with host neuropil.[47] Grafts lack the characteristic butterfly appearance of normal spinal cord but contain differentiated areas that resemble substantia gelatinosa.[24] This resemblance to normal spinal cord encourages the expectation that transplants might replace damaged populations of neurons and, with them, their normal connections. One additional way in which transplants of embryonic spinal cord may contribute to recovery of behavioral function has already been demonstrated in newborn hosts, where transplants have

been shown to rescue injured host rubrospinal neurons otherwise destined to die.[8] Whether transplants to spinal cord can also rescue axotomized neurons in adult hosts is currently being studied in several laboratories.

In newborn hosts, spinal cord transplants have the capacity to act as bridges that support the elongation of supraspinal axons into regions of intact spinal cord well below the level of injury. Both serotonergic axons originating in neurons in the brainstem raphe nuclei[6] and the axons of corticospinal neurons[7] traverse transplants placed into lesioned newborn thoracic spinal cord and establish projections within host spinal cord as far caudal as the lower lumbar levels. Animals with transplants perform better than littermates with lesions alone on several tests of locomotor function,[28] consistent with the idea that the axons that grow into host spinal cord caudal to the lesion contribute to the improved performance. If so, then transplants into newborn spinal cord have assisted in the recovery of complex behavior that requires not only the reconstitution of precise input by supraspinal neurons but also intact afferent control of these supraspinal neurons.

In adults, projections from host into transplant and from transplant into host are more modest, and damaged host axons do not traverse the full extent of the graft.[47] However, serotonergic axons from brainstem raphe nuclei,[47] axons from corticospinal neurons,[7] and primary afferent axons originating in dorsal root ganglion neurons[56] regenerate into transplants, whereas they are unable to elongate into spinal cord in the absence of a transplant (Fig. 14–1). Dorsal root ganglion axons not only establish synapses with neurons within transplants (Fig. 14–2), but these synapses resemble in several respects those formed by dorsal root ganglion axons within the dorsal horn of normal spinal cord.[23] The establishment of

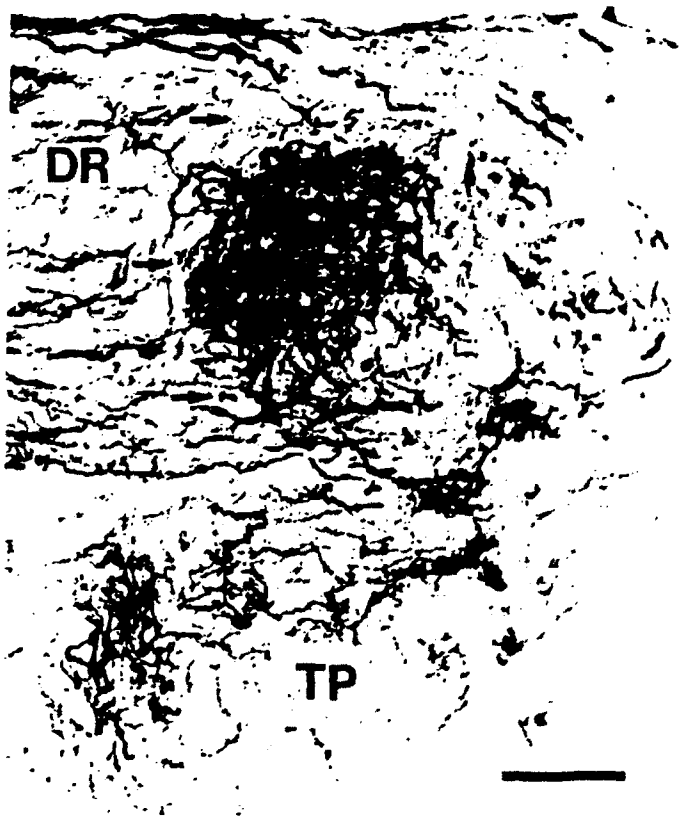

FIGURE 14–1. Sagittal section of the interface (*arrows*) between fetal spinal cord transplant (TP) and host dorsal root (DR) showing regenerated host dorsal root axons within the transplant. The dorsal axons have been labeled to demonstrate calcitonin gene-related peptide immunoreactivity, since this is a specific label for many of the primary different axons that terminate in the dorsal horn of normal spinal cord. (Bar = 100 μm.)

FIGURE 14-2. Electron micrograph showing synapses formed by host dorsal root axon which has regenerated into the fetal spinal cord transplant. A complex terminal makes asymmetric synaptic contacts (*arrows*) upon three different dendritic profiles (D). The dorsal root has been labeled by the direct application of wheat germ agglutinin-conjugated horseradish peroxidase. (Bar = 1 μm.)

morphologically normal synapses by an identified population of host neurons afferent to the transplant encourages the idea that relays of synaptically coupled neurons formed within transplants may allow the transplants to act as bridges across damaged spinal cord. Donor neurons in transplants have in fact been shown to send axons through the transplant into the peripheral nervous system of the host[56] (Fig. 14-3). Therefore it is at least possible that transplants of fetal spinal cord have the capacity to restore a damaged motor reflex arc as well as to stimulate or support the regeneration of axons otherwise unable to regrow. An important component of these transplants is thought to be the contribution of the glia, especially the astrocytes.

Prostheses of Fetal Astrocytes

A third strategy for using transplants to mediate behavioral recovery after spinal cord injury is to use prostheses prepared from immature astrocytes as bridges that will promote the regeneration of damaged central nervous system (CNS) axons. In the developing CNS, the surfaces of astrocytes and astrocyte precursors provide an important substrate for neuron migration[31] and the outgrowth of neuronal processes.[49,50] Injured adult axons may retain or reacquire

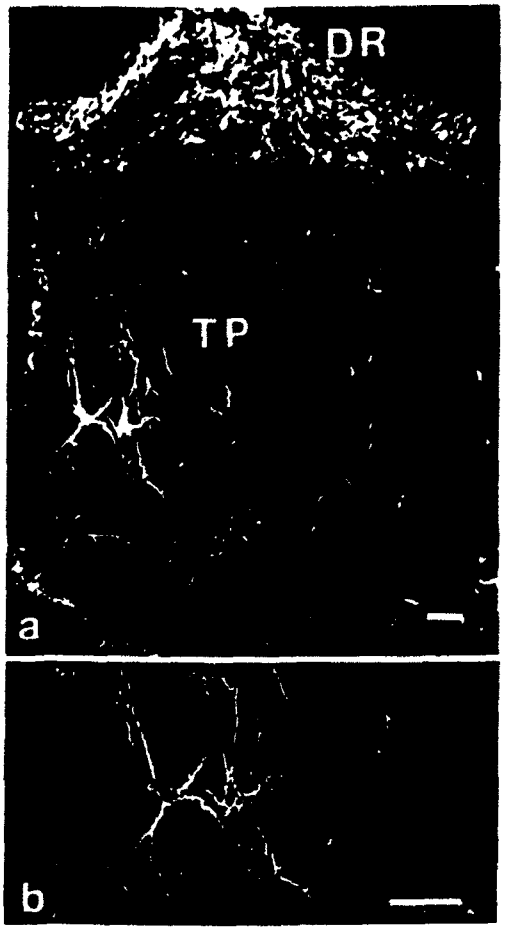

FIGURE 14-3. Dark-field photomicrographs of transverse sections through a fetal spinal cord transplant following injection of cholera toxin–conjugated horseradish peroxidase into the ipsilateral sciatic nerve *A*. Within the transplant (TP) are the cell bodies of neurons whose axons have grown out from the transplant and down the sciatic nerve of the host. Regenerated host dorsal root axons (DR) are also shown at the margin of the transplant. (Bar = 200 μm.) *B*, The labeled cell bodies are shown at higher magnification. (Bar = 100 μm.)

the capacity to growth along immature glia, since astrocytes isolated from newborn corpus callosum and trapped on a piece of Millipore filter can stimulate the regrowth of damaged corpus callosum axons and guide them across the site of injury to project into normal regions of termination.[53] In a first attempt to apply the same technique to the repair of the damaged spinal cord, the L-5 dorsal root was crushed approximately 2 to 3 mm from the dorsal root entry zone, and a prosthesis consisting of a pennant-shaped piece of Millipore filter was coated with astrocytes isolated from embryonic spinal cord and inserted into the spinal cord so as to direct the crushed dorsal root axons into the dorsal horn.[26] Control animals that received implants not coated with astrocytes showed an intense inflammatory reaction to the implant, along with hemorrhage and cavitation in the adjacent spinal cord and no ingrowth of regenerating axons into the dorsal horn. Animals that received implants coated with astrocytes showed a reduced inflammatory response and little hemorrhage or cavitation. In these animals, regenerated axons grew along the implant and terminated within the dorsal horn, some after first following an aberrant circumferential path around the dorsal horn. Although the experimental injury was mild compared with the massive tissue destruction seen in human spinal cord injury, and although the functional significance of the regeneration remains to be explored, the results of this experiment suggest that transplanted astro-

cytes can both reduce the necrosis that accompanies spinal cord injury and promote the regeneration of axons past the site of injury. Moreover, once past the site of crush, the elongating axons are able to recognize cues that enable them to return to their normal sites of termination within the dorsal horn.

SUMMARY

Transplants of embryonic CNS tissue have for many years contributed to the study of axon development and regeneration. Neurologists and neurosurgeons became interested when grafts were reported to provide a method of therapy for Parkinson's disease and potentially for other illnesses. Several features of Parkinson's disease made it a promising candidate for treatment by transplantation, and both fetal substantia nigra and adult adrenal medulla grafts have restored locomotor function in experimental models of parkinsonism. The mechanisms by which transplants improve function are debated, as is the role of transplantation in the treatment of patients with Parkinson's disease. Several strategies have also been proposed for using transplants to treat experimental spinal cord injury, including the use of transplants of brainstem monoaminergic neurons, transplants of embryonic spinal cord, and prostheses of embryonic astrocytes. The functional effects of transplants into spinal cord are in the early stages of investigation. However, transplants continue to provide insights into the mechanisms by which axons grow, and they represent a first step toward the development of a rational treatment of human spinal cord injury.

ACKNOWLEDGMENTS

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Differentiation of Substantia Gelatinosa-Like Regions in Intraspinal and Intracerebral Transplants of Embryonic Spinal Cord Tissue in the Rat

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The differentiation of intracerebral and intraspinal transplants of fetal (E_{14}–E_{15}) rat spinal cord was studied to determine the extent to which myelin-free zones in these embryonic grafts exhibit cytological features and immunocytochemical characteristics of the substantia gelatinosa (SG) of the normal spinal cord. Immunocytochemical staining with antiserum to myelin basic protein (MBP) revealed myelin-free areas of varying proportions within fetal spinal cord grafts. These regions were identified in both newborn and adult recipients regardless of whether donor tissue was grafted to heterotopic (intracerebral) or homotopic (intraspinal) sites. As in the SG of the intact spinal cord, the myelin-free regions consisted mainly of small (7–15 μm) diameter neurons. At the ultrastructural level, these cells were surrounded by a neuropil composed of numerous small caliber, unmyelinated axons and intermediate-sized dendrites. Synaptic terminals in these areas were primarily characterized by the presence of clear, round vesicles, although granular vesicles were occasionally found within these terminals. Immunocytochemical staining demonstrated met- and leu-enkephalin-, neurotensin-, substance P-, and somatostatin-like immunoreactive elements within these myelin-free areas. Thus, regions within embryonic spinal cord grafts undergo some topographical differentiation which parallels that of the normal superficial dorsal horn. The presence of SG-like regions illustrates the potential capacity of fetal spinal cord transplants for replacing some intraspinal neuronal populations at the site of a spinal cord injury in neonatal and adult animals. These graft regions may serve as a source of intersegmental projection neurons or establish an extensive intrinsic circuitry similar to that seen in the normal SG. In addition, the definition of these areas provides a useful model to study the innervation patterns of host axons that typically project to the substantia gelatinosa of the normal spinal cord. © 1989 Academic Press, Inc.

INTRODUCTION

Intracerebral grafts of fetal CNS (central nervous system) tissue have been shown to compensate for a variety of functional deficits in experimental animal models, through the replacement of neuronal circuitries or neurotransmitters or by the production of neuronotrophic substances within the host brain (reviewed in (5, 7, 15, 46). In recent years, there has been some enthusiasm for the application of fetal CNS tissue transplantation techniques to the problem of spinal cord injury (e.g. (3, 9, 10, 13, 14, 21, 34, 39)). Together, these reports indicate that intraspinal transplantation results in the survival and integration of embryonic donor tissue in both neonatal and adult recipients. In this context, embryonic CNS tissue may be used to promote repair by bridging the lesion and thus providing a favorable matrix through which axons can grow (8, 31, 36). In other cases, fetal grafts may be used to replace supraspinal inputs to motoneuron pools caudal to the lesion (6, 10, 31, 32, 34).

A third strategy for functional repair involves the introduction of fetal neurons into the lesion site. The integration of transplanted tissue with the host neuronal elements could allow for the formation of a neuronal relay by establishing intersegmental connections (e.g. (31, 36)). In particular, homotopic grafts could serve as a source of specific intraspinal neuronal populations with an inherent potential for integrating with synaptic circuits above and below the injury. Therefore, recent attention has been given to the intraspinal transplantation of embryonic spinal cord tissue into both acute and chronic lesion sites in the rat spinal cord (21, 38, 39, 40). The survival, host–graft integration, and connectivity of such homotopic grafts have been examined (21, 39, 51). In addition, some degree of homotypic differentiation has been indicated by studies in which fetal spinal cord transplants were placed into intracerebral or intraspinal

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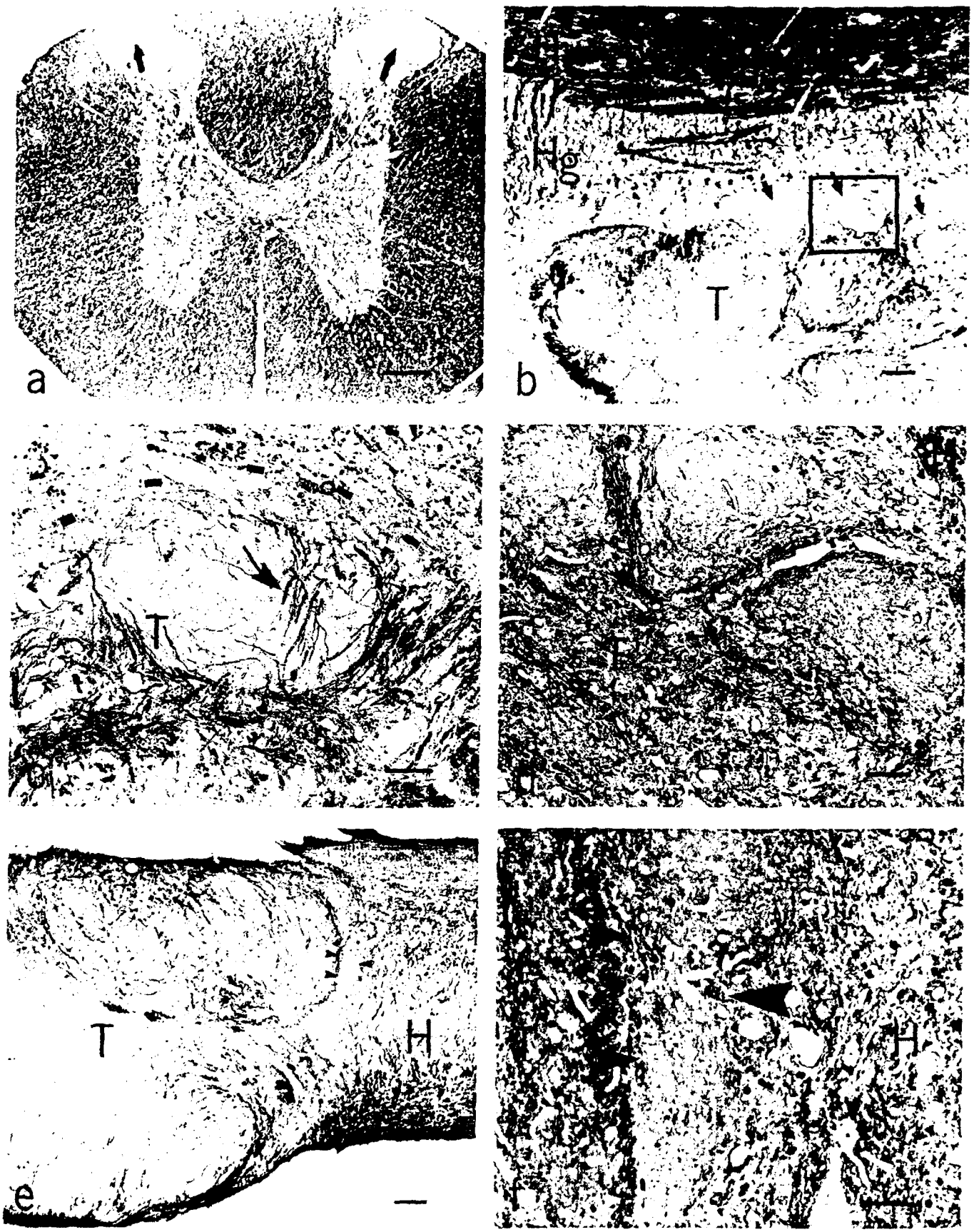
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cavities (37-40). In these preliminary investigations, distinct myelin-free regions were observed in matured grafts, leading to the hypothesis that these unmyelinated areas corresponded to the superficial dorsal horn—especially the substantia gelatinosa (SG)—of the normal spinal cord.

In the present study we have examined in more depth the myelin-free regions of fetal spinal cord grafts to determine the extent to which these areas develop cellular and ultrastructural characteristics of the normal SG. In addition, immunocytochemistry was used to determine whether some peptidergic elements normally seen in the mature superficial dorsal horn are also seen in the myelin-free regions. In order to evaluate the intrinsic differentiation of this region under a variety of conditions, grafts were placed into spinal or supraspinal levels and into newborn and adult recipients.

MATERIALS AND METHODS

Animal Groups

Thirty-nine adult (200–300 g) Sprague–Dawley (Zivic-Miller, Allison Park, PA) rats of either sex received either intracerebral (N = 15) or intraspinal (N = 24) implants of E_{14} or E_{15} spinal cord tissue (Day E_0 = day of insemination). A total of 15 newborn rat pups (less than 72 h after birth) received intraspinal transplants of embryonic spinal cord tissue.

Surgery

The surgical procedures used for intracerebral and intraspinal transplantation were identical to those previously described (8, 39, 41). In brief, adult rats were anesthetized with ketamine (60–70 mg/kg) and xylazine (10 mg/kg) and administered a preoperative dose of penicillin (Wyeth, Bicillin C-R; approximately 60,000 U, im). Intracerebral transplants were placed into a posterior cortical cavity made by aspirating portions of the occipital cortex and hippocampal formation at the level of the superior colliculus (48). After hemostasis was achieved, the donor tissue was placed on the vascular bed of the choroid fissure. The remainder of the cavity was filled with gelfoam, the craniotomy was repaired with bone wax, and the scalp incision was closed with surgical wound clips.

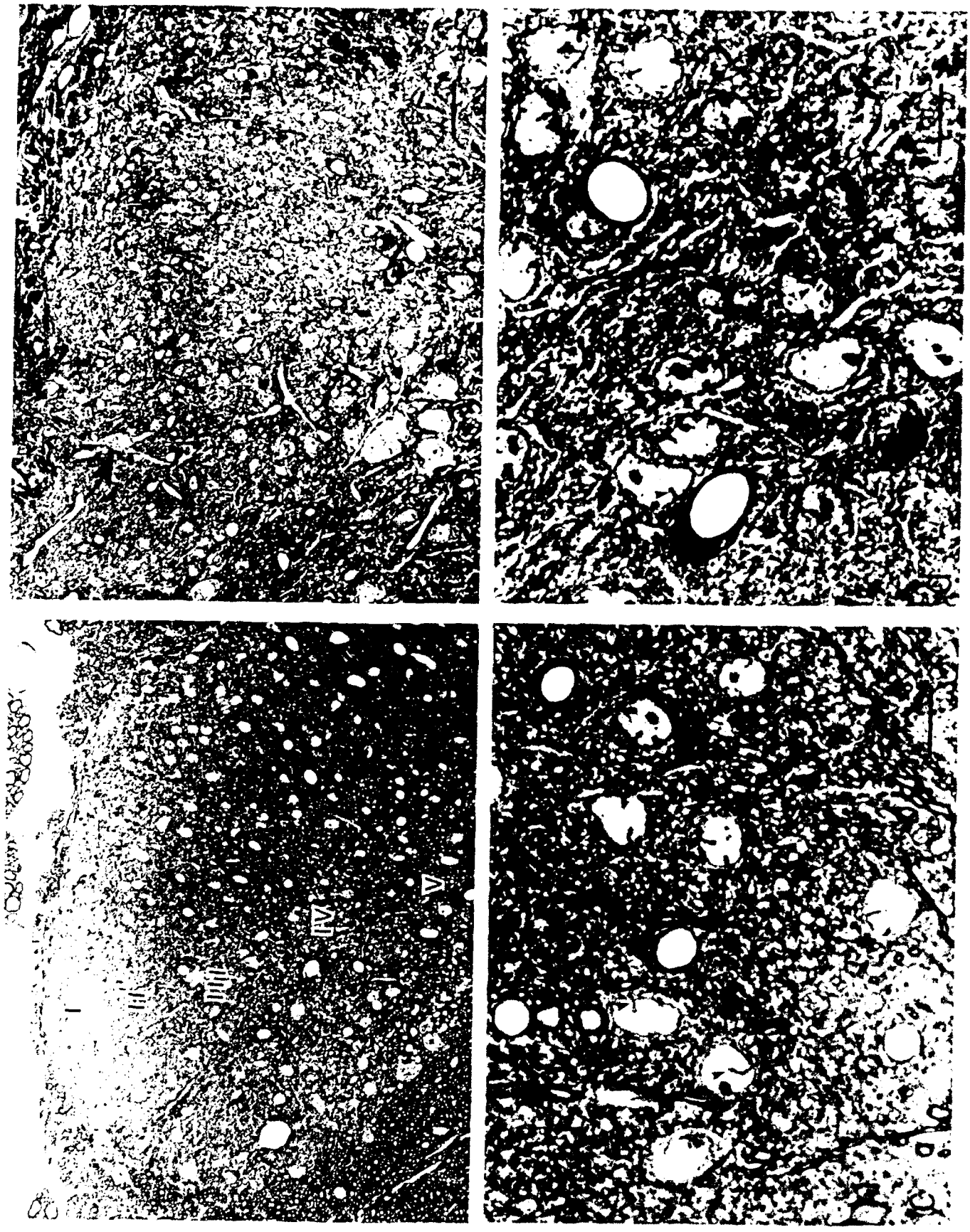
For intraspinal transplants into adult animals, laminectomies were performed at cervical, thoracic, or lumbar spinal levels. The grafts were introduced into an aspirative lesion cavity, 3–4 mm in length, consisting of either an extensive dorsal funiculotomy or a transverse hemisection. Newborn rats were anesthetized by hypothermia and received a laminectomy just caudal to the blood supply of the dorsal fat pad (T_5–T_8). Transplants were then placed into a transverse hemisection of the spinal cord measuring 3 mm in length. After placement of the grafts in both the neonatal and adult recipients, the dural incision was closed as previously described, the overlying muscles were sutured in layers, and the skin incisions were closed with wound clips.

For each transplantation session, pregnant Sprague–Dawley rats were maintained under deep chloral hydrate anesthesia (400 mg/kg) and fetuses were removed as donor tissue was required. Fetuses were transferred to sterile tissue culture medium (Dulbecco's modified essential medium) in which the spinal cord was isolated. The fetal meninges (except for the pia mater) were then peeled away as a continuous sheath, resulting in the detachment of spinal ganglia. Whole segments of donor tissue, 3–4 mm in length, were then introduced into the lesion cavities as previously described. To determine if the differentiation of SG regions was dependent upon afferent innervation from surrounding host tissue, two intraspinal transplants (adult recipients) were intentionally isolated from the recipient cord by cografting pieces of the embryonic meninges which established a connective tissue capsule around the donor tissue.

Light and Electron Microscopy

At random intervals from 1 to 4 months after transplantation, 10 intracerebral and 15 intraspinal recipients (12 adult, 3 newborn) were anesthesized with a lethal dose of sodium pentobarbital and perfused through the heart with 0.9% NaCl followed by 5.0% glutaraldehyde and 4.0% paraformaldehyde in 0.1 M phosphate buffer. One adult recipient was sacrificed after 6 months to determine if any obvious changes in SG development occurred at longer survival times. Following the perfusion, graft-containing segments were then excised and divided into several transverse or longitudinal slices. The specimens were subsequently osmicated, dehydrated, and embedded in Epon for plastic thick sectioning. Regions of the grafts classified as "SG-like" were

FIG. 1. Identification of myelin-free areas. (a) Pattern of PAP staining with antiserum against MBP in a transverse section of a normal rat thoracic spinal cord, showing the myelin-free regions in the superficial dorsal horn (arrows). (b) Anti-MBP staining in a 2-month intraspinal transplant (T) and surrounding host intermediate gray (Hg) and lateral white matter (Hw) (horizontal section). Three myelin-free patches are seen at the edge of the transplant (arrows). (c) Enlargement of boxed region in (b). Myelinated axons (arrow) cross through this otherwise unmyelinated area. Dotted line highlights the host/graft interface. (d) Toluidine-blue-stained 2-μm section of another intraspinal graft showing myelinated regions containing larger neurons and unmyelinated patches similar to those in (c). (e, f) Adjacent saggital sections from an intraspinal transplant illustrates the correspondence between an MBP-negative region and an enlargement of the same area (arrowheads) which is occupied by small cells and processes. Magnification bars in (a), (b), and (e) = 200 μm; and in (c), (d), and (f) = 50 μm.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trimmed, and thin sections were surveyed at the ultrastructural level.

Immunocytochemistry

At similar intervals, the remaining intracerebral, and 22 of the intraspinal graft recipients were perfused 4.0% paraformaldehyde in 0.1 *M* Sorenson's phosphate buffer (*p*H 7.4). The remaining 2 intraspinal recipients were sacrificed at 8 and 9 months, respectively. Graft-containing specimens were excised and prepared for either cryostat (15 μm) or Vibratome (25–40 μm) sectioning. Specimen blocks used for immunofluorescent staining were transferred through a graded series of 10, 20, and 30% sucrose in 0.1 *M* phosphate buffer at 4°C for at least 2 h for each step. The blocks were frozen after a final overnight rinse in 30% sucrose. Resulting sections were then processed for either the indirect immunofluorescence or peroxidase-antiperoxidase (PAP) method (49) using primary antisera raised in rabbits against met- and leu-enkephalin (met- or leu-ENK), neurotensin (NT), substance P (SP), or somatostatin (SOM) obtained from Immunonuclear Corp. (Stillwater, MN). Care was taken to maintain sections in serial order; the first and the last sections in a given series were stained with antiserum to Myelin Basic Protein (MBP; provided by Dr. L. F. Eng, Palo Alto, CA, VA Medical Center) to identify the myelin-free regions under investigation. The intervening sections were stained with the antisera to some or all of the peptides listed above. In addition, sections from the thoracic spinal cord of normal rats were processed with the same techniques to illustrate normal characteristics and staining patterns within the substantia gelatinosa. Two intracerebral graft recipients received injections of colchicine (20 μl, 10 mg/ml, Sigma) directly into the transplant 48 and 24 h prior to sacrifice to reveal neuronal cell bodies.

For PAP staining, tissue sections were incubated overnight at room temperature in primary antiserum diluted to 1:2000 with a solution of 0.3% Triton X-100 in phosphate-buffered saline (PBS) containing 10% normal goat serum. The sections were washed several times in PBS and then incubated in goat anti-rabbit IgG (Cooper Biomedical or Sternberger-Meyer) diluted 1:10 with the primary antiserum diluent for 45–60 min at room temperature. After a rinse with PBS, the sections were incubated for 45 min in rabbit PAP (Cooper Biomedical or Sternberger-Meyer) diluted 1:50. Following several washes in PBS and 0.05 *M* Tris buffer (pH 7.6), the immunocytochemical reaction product was developed in a 0.05 *M* Tris buffer solution containing 0.05% 3,3′-diaminobenzidine hydrochloride (Sigma), 0.3% H_2O_2. Antibody specificity was verified by the absence of immunoreactive elements when primary antibody was replaced with antiserum preabsorbed with 50 μg/ml of peptide.

For immunofluorescence visualization of met-ENK and NT, frozen sections were affixed to slides and washed several times in PBS at room temperature. The slides were then incubated in primary antiserum either to NT (1:500) or met-ENK (1:500 or 1:1000) at 4°C overnight in a moist chamber. The sections were again washed several times with PBS and incubated in fluorescein-isothiocyanate (FITC)-conjugated to goat anti-rabbit IgG (Miles/Sigma) at a dilution of 1:50 for 30 min at 37°C. Finally, the sections were washed and coverslipped with *n*-propyl gallate.

RESULTS

Light Microscopy and Cytological Characteristics

Sections of normal rat spinal cord, when stained with antiserum directed against myelin basic protein (MBP), exhibit a characteristic pattern of myelin distribution. Specifically, the long fiber tracts appear densely stained, while a moderate staining reflects the presence of myelinated fibers coursing throughout most of the central gray matter. The most striking feature of this preparation, as also seen with conventional myelin stains, is the superficial dorsal horn. This corresponds to the cytoarchitecturally defined substantia gelatinosa (SG), which stands out against the background of intense myelin immunoreactivity due to the paucity of myelinated axons in this region of gray matter.

Fetal spinal cord transplants were stained with anti-MBP to examine the differentiation of the grafts. All of the transplants examined with this technique were heavily myelinated as indicated by areas of very dense staining. In addition, large regions exhibiting moderate immunoreactivity similar to normal gray matter were evident. Also, the transplants usually contained one or more areas which were conspicuous due to the marked absence of MBP-like staining (Figs. 1b, 1c, and 1e). These myelin-free areas typically assumed a convoluted configuration within the grafts, and appeared as either

FIG. 2. Cytology of the normal SG and SG-like regions in FSC grafts. (a, c) Two-micrometer toluidine blue-stained transverse sections from the normal adult rat dorsal horn (laminae I–V) and the SG (lamina II). Neurons within the SG are tightly packed and contain large nuclei with prominent indentations (arrowheads). Most of the unmyelinated processes in this region course perpendicular to the plane of section. An unmyelinated region from an E_{14} intracerebral graft (b) strongly resembles the normal dorsal horn (in this figure, the host tissue cannot be seen). Larger neurons, such as the one here (arrow), were rarely found. At a higher magnification (d), the cells within the SG-like regions resemble their counterparts in the normal SG. However, the processes lack the longitudinal orientation characteristic of the normal dorsal horn. Magnification bars in (a) and (b) = 50 μm; and in (c) and (d) = 10 μm.

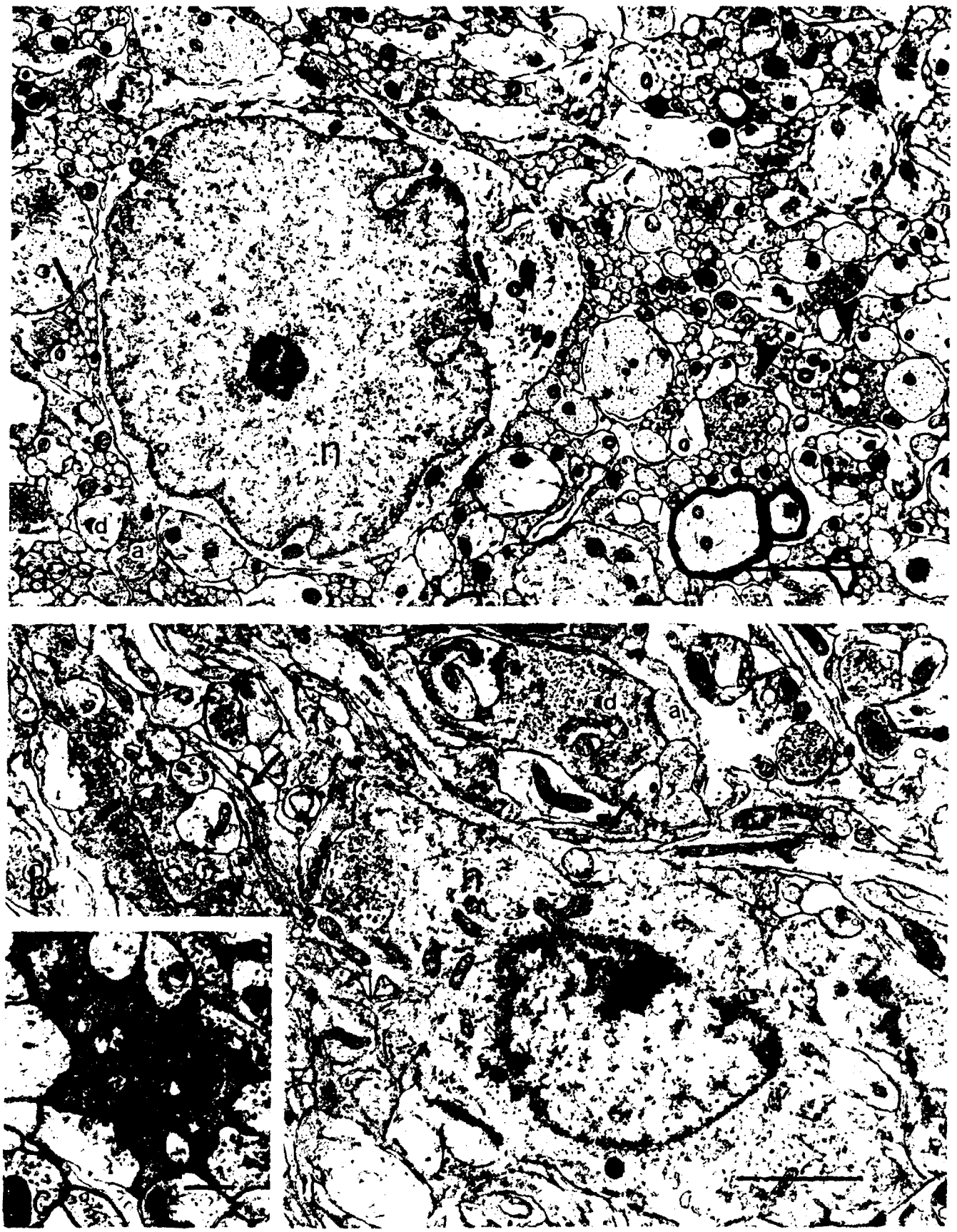

FIGURE 3

single or multiple patches or long strips of neuropil depending upon the plane of section. In many cases, these regions were located near the periphery of the grafts (Fig. 1b); however, they were not always restricted to the margins of the transplants, as some myelin-free areas were located more centrally (Fig. 1e). Myelinated axons frequently curved along the surfaces of these unstained regions, and in some cases, a few anti-MBP-stained processes traversed the myelin-free zones in a radial fashion, reminiscent of the pattern of myelinated primary afferents projecting to deeper layers of the gray matter in the normal spinal cord (Fig. 1c).

With the perspective derived from MBP-stained grafts, examination of toluidine blue-stained sections of FSC transplants revealed areas that appeared to correspond with the MBP-free regions. These 2-μm sections demonstrated extensive myelination within all of the grafts studied, as well as numerous myelin-deficient areas embedded within the dense matrix of myelinated donor tissue. These regions developed regardless of whether the grafts were placed into intracerebral or intraspinal cavities or into immature or mature recipients.

To determine whether the myelin-free areas identified in various grafts by immunohistochemistry or in plastic thick sections were indeed equivalent, one specimen was processed so that for each MBP-stained section there was an adjacent 100-μm section embedded in plastic. In this specimen, all of the zones that failed to show MBP immunoreactivity, but were surrounded by myelinated fascicles, were closely in register with a homogenous unmyelinated area in the adjacent toluidine blue-stained section (Figs. 1e and 1f).

Further examination of these regions in plastic sections revealed that similarities between the myelin-free areas of FSC grafts and the normal superficial dorsal horn included more than the common absence of myelinated fibers. As in the normal substantia gelatinosa (Figs. 2a and 2c), the unmyelinated zones of matured transplants consisted of numerous small cells (7–15 μm) having a rather thin rim of cytoplasm surrounding a prominent nucleus. The nuclei of these cells, as of those in the normal superficial dorsal horn, were round or oval and often exhibited large clefts (cf. Figs. 2c and 2d). These cells differed considerably from the larger neurons (14–50 μm in diameter) that were found within the myelinated regions of the transplants. The presence of larger neurons within the unmyelinated areas was very rare.

In contrast with the fact that the normal SG and myelin-free graft areas shared many common features, there were also some obvious differences in the distribution of cells and neuritic processes. Whereas in the intact spinal cord, SG neurons can be differentiated into two layers (i.e., IIi and IIo, Refs. (34) and (49)), no obvious cytoarchitectural lamination was seen in the transplants. It was interesting to note that some of the larger cells within the myelinated regions were closely apposed to the border of the unmyelinated zones. These general cellular relationships were similar to the approximation of lamina III and IV neurons with the normal SG. In addition, many small, circular neuritic profiles were seen in transverse sections of the normal SG, thus reflecting their orientation parallel to the longitudinal axis of the spinal cord. In contrast, the neuritic processes in the myelin-free areas of the grafts seemed more randomly organized with many longitudinally sectioned profiles being seen.

Electron Microscopy

Neurons within the normal substantia gelatinosa were generally spheroid (Fig. 3a) or fusiform (Fig. 3b) in shape. The perikarya ranged from 8–20 μm in diameter, and, as seen with the light microscope, they usually contained a large nucleus within a narrow rim of cytoplasm. These cells were embedded in a neuropil that primarily consisted of tightly packed small, unmyelinated axons and small to intermediate-sized dendrites. Nearly all of the synapses identified within the SG were axodendritic in nature (Fig. 3b). In addition, numerous large glomerular complexes were evident in these normal sections (Figs. 3a and 3c).

A survey of the unmyelinated regions within FSC transplants revealed many of the same characteristics (Fig. 4). The neurons in these areas were of comparable size and contained large nuclei with prominent indentations. The cells were closely spaced, and were surrounded by compact neuropil consisting of small axons (0.1–0.3 μm) and intermediate-sized dendrites (0.4–1.6 μm). Occasionally, small bundles of unmyelinated processes were seen which resembled the fascicles in the normal SG. However, most of the axons and dendrites were more randomly oriented. Except for an occasional swollen neuritic profile containing lysosomes and degenerating mitochondria, axonal and dendritic processes did not display any irregular cytological characteristics. In a few transplants, hypertrophic astrocytic processes were observed (Fig. 5b), but in general, fibrous glial processes were relatively uncommon in both the normal SG and transplant myelin-free areas.

FIG. 3. Ultrastructure of the normal SG. (a) Transverse section contains a neuronal cell body (n) and the compact neuropil containing abundant axodendritic synapses (ad) interspersed with longitudinal bundles of unmyelinated processes (arrows). These fascicles are more difficult to discern in the oblique section in (b). Large glomerular complexes associated with primary afferent terminals were often found within these regions (large arrowheads in (a), also (c)). Filamentous glial processes (*) were rarely encountered within the neuropil of these normal specimens. Magnification bars in (a) and (b) = 2.5 μm; and in (c) = 0.5 μm.

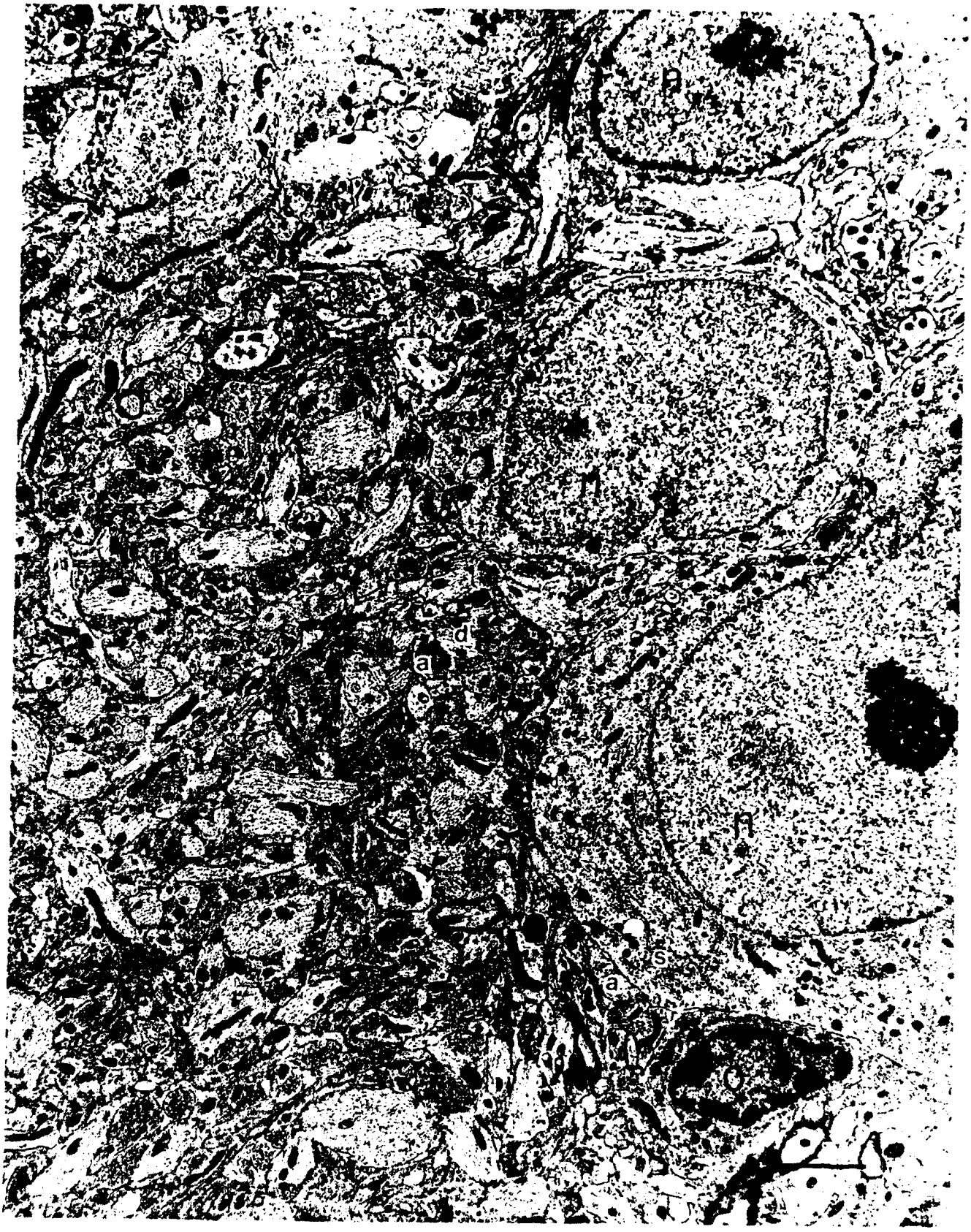

FIG. 4. Low power electron micrograph of an SG-like region in an E_{14} intracerebral transplant. The small neurons (n) were often very close together, and occasionally, an oligodendrocyte (o) was encountered at the periphery of an SG-like area. An axodendritic (ad) synapse and an axosomatic (as) synapse are shown. Axonal and dendritic processes assumed a variety of orientations, and appeared to lack some of the organization of the normal spinal cord. Magnification bar = 2.5 μm.

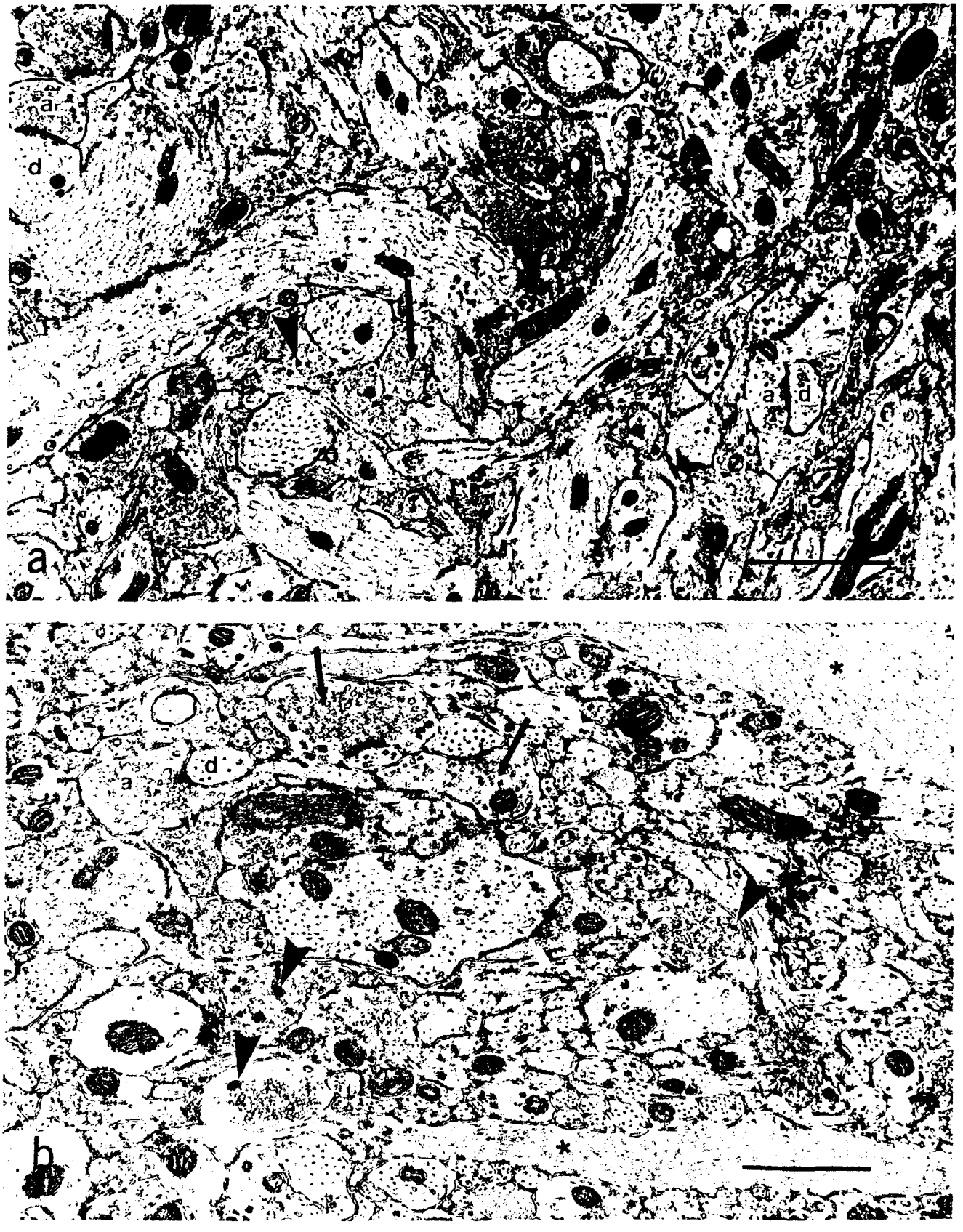

FIG. 5. Higher magnification of SG-like regions in a 2-month intracerebral (a) transplant and a 6-month intraspinal (b) transplant. The vast majority of synapses were axodendritic (ad), and vesicles were usually clear (agranular) and round (arrows), although flattened (double arrowhead) and dense cored (large arrowheads) vesicles were also observed. In a small number of the grafts examined, large filament filled astrocytic processes were present (*). Magnification bar in (a) = 2 μm; and in (b) = 1 μm.

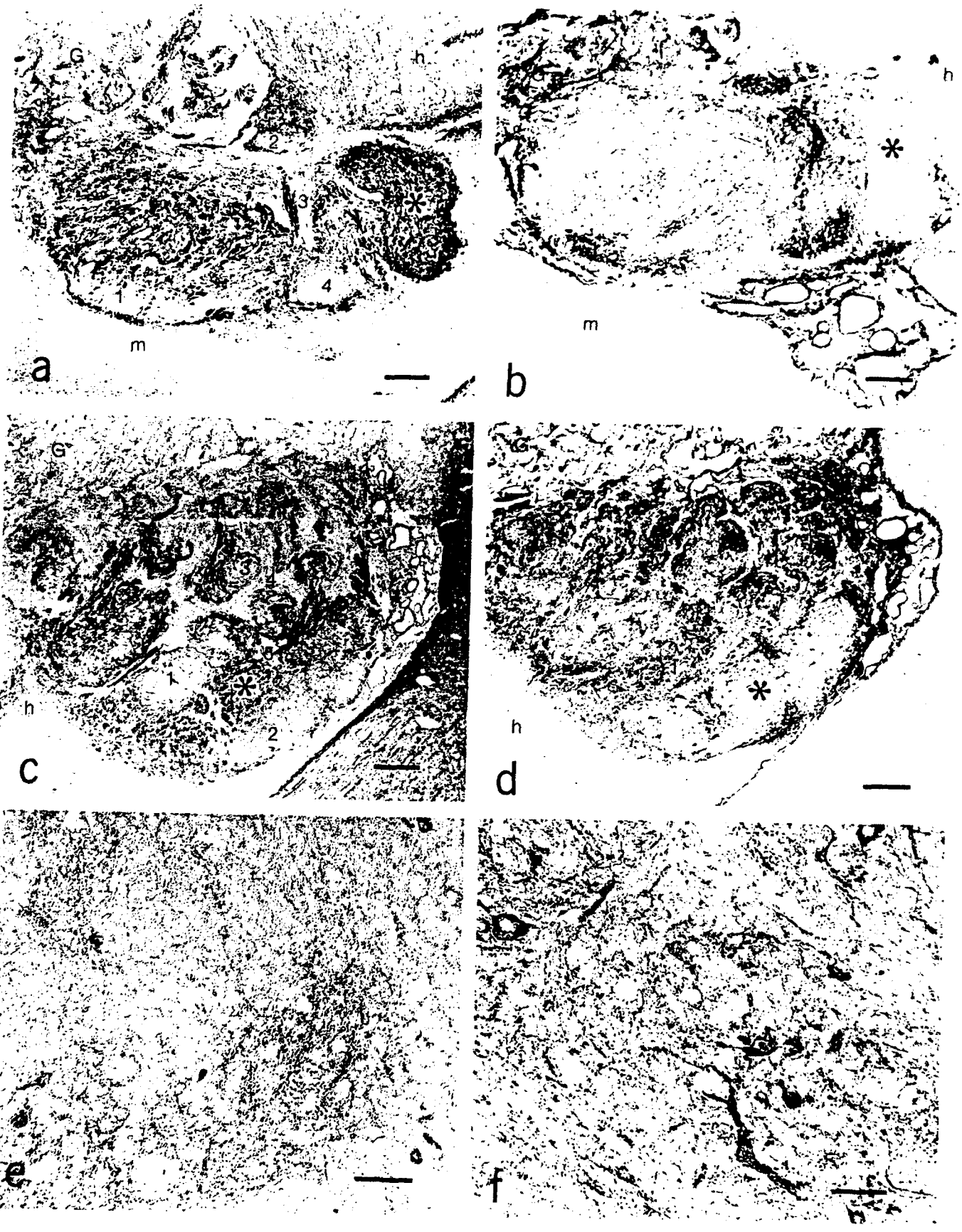

FIGURE 6

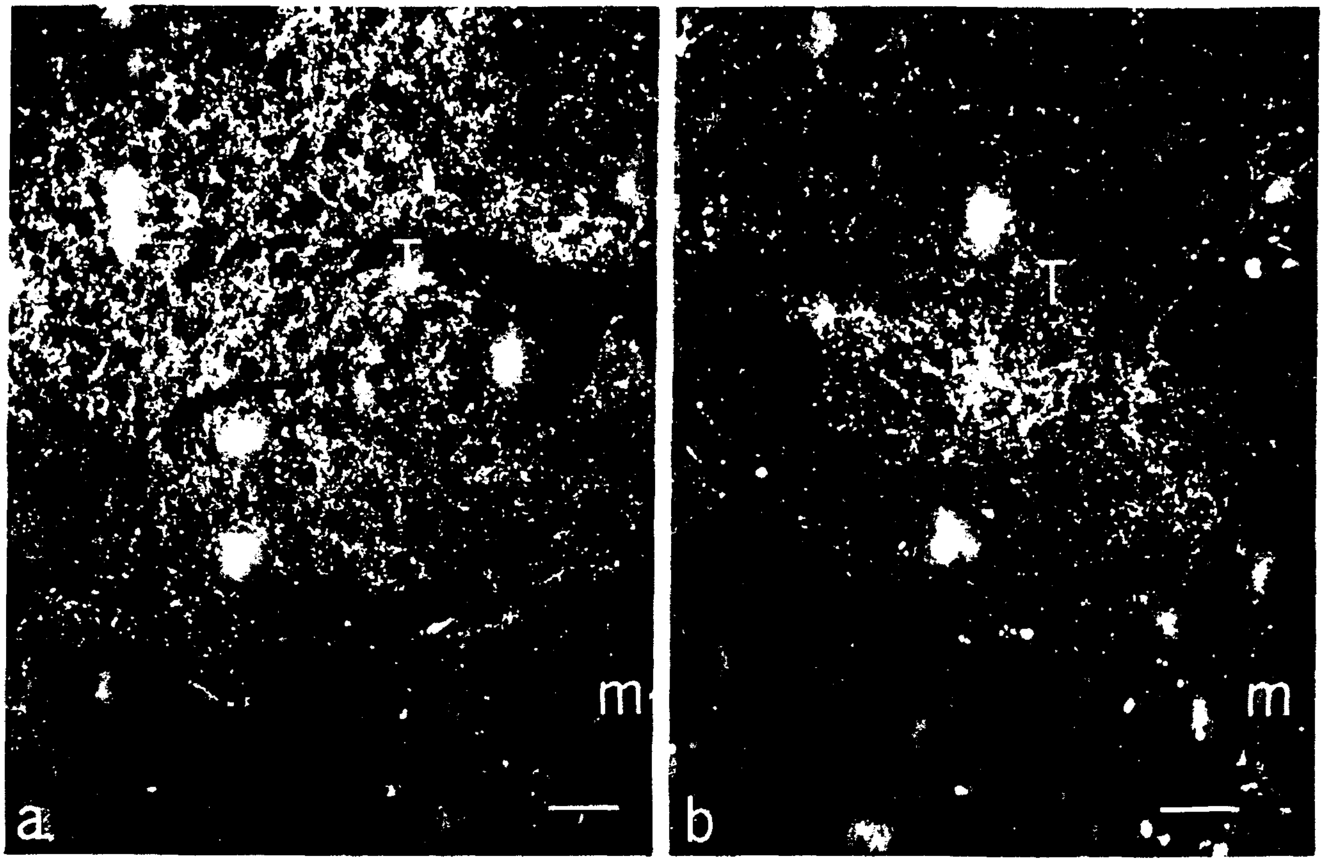

FIG. 7. Immunofluorescence staining of an intracerebral transplant with anti-met-ENK (a) and anti-NF (b). Sections are in the same orientation as those in Fig. 6, with the transplant (T) at [illegible] top of the figure apposed to the [illegible] brain region [illegible]. ENK-like immunoreactivity is dense, and extends over a wider area [illegible] the graft, while [illegible] patch of dense NF-like activity is restricted [illegible] smaller region. In addition [illegible] the heavy patches of fiber staining which corresponded to a myelin-free region (illustrated) a sparse fiber was [illegible] the transplant [illegible]. Magnification bars = 10 μm.

The vast majority of synaptic contacts within the unmyelinated regions were axodendritic (Figs. 4 and 5), with axoaxonic synapses occasionally being present as well. In addition, a rare axosomatic synapse could be found in normal and graft myelin-free areas. The boutons within the myelin-free regions usually contained aggregates of small, agranular vesicles (Figs. 5a and 5b). Most of the vesicles were round, rather than flattened, and some contained small dense-cored vesicles. Similar presynaptic structures were also seen in the normal SG. However, the scalloped terminals and related glomeruli characteristic of normal primary afferent innervation of the SG were not found within the grafts examined in this study.

Immunocytochemistry

Adjacent sections of either intracerebral (Figs. 6 and 7) or intraspinal (Fig. 8) transplants were stained with immunocytochemical markers to determine if those regions, defined by the absence of myelin, contained pep-

FIG. 6. Correspondence between MBP-negative and ENK-like staining [illegible]. MBP [illegible] ENK [illegible] two intracerebral grafts [illegible] months after [illegible] [illegible]

normal substantia gelatinosa. These general criteria, though intriguing, do not provide sufficient proof of the exact nature of the myelin-free areas in the grafts. In this context, it is well recognized that other characteristics make this region distinct from the rest of the gray matter in the intact spinal cord. In particular, the abundance of small cells led Rexed (43) to make the distinction of lamina II of the cat spinal cord. More recent studies of the ultrastructure of this region have also served to define the types of processes and synaptic profiles that distinguish the substantia gelatinosa (lamina II) from the surrounding marginal layer (lamina I) and nucleus proprius (lamina III) (35, 50). Additional identifying features have been noted through the use of immunocytochemical techniques, which have demonstrated a variety of peptide-containing cells and fibers (reviewed in (17, 22, 26, 44). With these characteristic cytological and immunocytochemical features as a basis for comparison, the present study has provided additional evidence in support of the organotypic differentiation of substantia gelatinosa-like regions in transplants of fetal spinal cord tissue. In FSC transplants examined with light and electron microscopy, we have observed cytological and ultrastructural features which closely resemble those found in the normal SG. In addition, a major finding of this study is the demonstration of a frequent correspondence between these myelin-free areas and regions of dense immunoreactivity obtained with antibodies to several peptides which are normally associated with the substantia gelatinosa. At the same time, we have noted some interesting discrepancies that may reflect altered developmental mechanisms following transplantation, as well as some implications of how these grafts may be used to study aspects of axonal connectivity in relation to spinal cord injury and repair.

Myelin-Free Areas and Peptidergic Elements

Using antibodies to met- and leu-enkephalin, neurotensin, substance P, and somatostatin, we have noted in mature FSC transplants the presence of defined patches of dense immunoreactivity which resemble staining patterns in the superficial dorsal horn. In addition, we have identified some ENK-containing cells within FSC grafts both with and without colchicine treatment.

Similar discreet areas of dense immunoreactivity to anti-ENK have been observed in previous studies of FSC grafts transplanted *in oculo* (4), and a more comprehensive analysis of the peptidergic composition of these grafts has been recently reported (20). In the latter study, comparisons were made between the patterns of immunoreactivity to eight neuropeptides in FSC grafts *in oculo* and the distribution of the same peptides in the normal spinal cord. The results indicated the presence of regions of dense immunoreactivity following staining with antibodies to peptides intrinsic to the SG, and further revealed some degree of overlap in their distributions. These studies by Henschen *et al.*, in combination with our present findings, confirm the differentiation of peptide-containing regions within FSC grafts. The correspondence of some of these regions with myelin-free areas, as identified with anti-MBP staining in this study, further underscores the proposed homology of these regions with the substantia gelatinosa.

The identification of these myelin-free regions with antibody staining against MBP has provided the basis for a comparison of the patterns of peptide staining as they relate to other features that characterize the normal substantia gelatinosa. This comparison sets the stage for further studies concerned with the development of appropriate synaptic relationships between peptidergic neurons in FSC transplants.

Developmental Implications

The observation that fetal spinal cord tissue can exhibit some degree of organotypic development is consistent with other studies showing that transplants of embryonic brain regions can achieve cytoarchitectural and ultrastructural characteristics corresponding to those of the homologous areas in the intact CNS (e.g. (2, 3, 24, 25, 27)). Fetal spinal cord tissue has also been shown to exhibit some cytoarchitectural or immunocytochemical characteristics resembling the normal dorsal horn when grown in tissue culture (30, 47) or *in oculo* (19). In this regard, the present findings also show that the differentiation of the myelin-free areas occurs irrespective of the graft site or maturity of the recipient CNS.

The presence of a dorsal horn component in FSC grafts examined in this study may be related to the developmental timing of this region of the spinal cord. Previous studies of spinal cord histogenesis in the rat (1, 33) have indicated that there is a peak at approximately E_{15}–E_{16} in the generation of neurons which ultimately comprise the dorsal horn. These cells then migrate with the majority of neuroblasts reaching the presumptive dorsal horn region 2 days later. The maturation of the SG-like areas in fetal spinal cord grafts must therefore occur after transplantation, since donor tissue in our experiments was obtained at E_{14}–E_{15}. These considerations pertaining to cell birthdates and onset of migration also suggest that the clustering of small neurons into the SG-like regions may be due to the persistence of intrinsic recognition cues which influence the aggregation of these cells during normal development. These intrinsic cues are retained whether the graft is placed into homotopic or heterotopic sites, or when it is isolated from host afferent inputs *in situ* (intraspinal isolates) or *in vitro*.

Anomalous Features of the Substantia Gelatinosa-Like Regions

While many features of the myelin-free regions of FSC grafts reflect a homology with the normal SG, it

must be emphasized that the correspondence was not perfect. Our observations have also identified several aspects of these areas which represent a departure from the normal organization of the mature superficial dorsal horn. For example, the myelin-free graft regions lack the precise orientation and formation of a dorsolateral cap, the parallel longitudinal arrangement of neuronal processes, and the definition between the outer and inner layers of lamina II observed in the normal SG. It is likely that some of these differences are related to specific aspects of the grafting procedure, such as the initial orientation of the graft tissue, donor age, and changes in the precise timing of developmental cues (24). In addition, the topography of some of these transplants may be distorted, and therefore contribute to the organizational differences observed. The differentiation of organotypic regions within an abnormal cytoarchitectural framework has also been observed in other types of fetal CNS transplants (e.g. (29)).

Another factor that may contribute to the atypical features observed here is the deafferented state of the transplant. As noted in our electron microscopic results, these SG-like graft regions lacked the synaptic terminals characteristic of primary afferent innervation, and in many ways resembled the ultrastructure of a deafferented newborn or adult dorsal horn (12, 23, 42). In addition, the developing grafts may also be lacking any descending modulation present in the normal spinal cord. Therefore, rather than being indicative of aberrant development, the atypical features recorded may instead reflect the normal development of SG-regions in the absence of afferent input. Such a situation would not only alter the patterns of migration and lead to cytoarchitectural differences, but might also prevent the normal expression of neuropeptides and neurotransmitters.

Along these lines, we also noted some subtle differences in the pattern of peptide cell staining in the FSC grafts. In particular, we observed neurotensin-like cells and fibers throughout the transplant. This contrasts with the normal spinal cord, in which NT-containing fibers and cells are found only in the superficial dorsal horn (45). Similar results were found recently with regard to other peptides in cortical and spinal grafts that developed *in oculo* (16, 20). Taken at face value, the results seen *in oculo* suggested that the disturbed patterns of peptide staining might be attributed to the isolation of these transplants from the environment of the CNS. However, our present findings indicate that some alterations in peptide expression exist even in well-integrated grafts grown in homotopic locations in the CNS.

Implications for Repair

The functional role of cells in the mature substantia gelatinosa of the spinal cord is a topic still under intense speculation (reviewed in (11, 52)). The termination of unmyelinated primary afferent fibers in this region provides the basis for theories as to its role in the modulation and gating of pain and reflex functions (28, 52). Szentagothai (50) used Golgi stains and degeneration techniques to reveal the morphology and projection patterns of SG neurons, and proposed that the SG is primarily a closed system, dominated by local projection neurons that extend no more than two or three segments. More recent evidence obtained with axonal tracing techniques has shown that at least some of these SG neurons can project as far as the medulla (18) and thalamus (53). Therefore, the differentiation of SG areas within FSC transplants suggests a source of intrinsic modulatory cells as well as some projection neurons that may play a role in the formation of a neural relay for somatosensory information.

It is not known which region of the embryonic neuroaxis is best suited to restore function in the injured spinal cord. However, successful repair of damaged neural networks may require the reconstruction of certain suprasegmental and intraspinal circuits. Our recent studies have shown that in some instances, homotopic grafts are innervated in varying degrees by host serotonergic and primary afferent fibers (8, 38, 39, 51). Both of these axonal systems, as well as many other identifiable fiber populations normally project to the SG. Because these SG-like areas are easily identified within FSC transplants, and because the afferent innervation of the normal SG is well characterized, this transplantation model should provide a valuable opportunity for testing the ability of host axons to recognize regions of these grafts with similar cytological and peptidergic characteristics to the SG of the normal spinal cord. Such information can be useful in further understanding the potential of the grafts to reconstruct specific circuitries in the injured spinal cord.

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CHAPTER 27

Enhancement of adult dorsal root regeneration by embryonic spinal cord transplants

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Introduction

Transplants of embryonic spinal cord have been shown to survive in the spinal cord of adult (Patel and Bernstein, 1983; Reier et al., 1986a) and newborn (Bregman and Reier, 1986) host rats and to develop regions that resemble the substantia gelatinosa of normal adult spinal cord (Reier et al., 1986b). Homotopic grafts may therefore replace specific circuits and eventually provide a strategy for repairing the damaged spinal cord. In order to understand the mechanisms by which transplants may mediate repair, it is of interest to determine whether transplants can receive projections from neurons that terminate in normal spinal cord and whether the targets of these projections resemble their normal counterparts. The central processes of dorsal root ganglion (DRG) neurons offer advantages for these studies since their terminations in normal spinal cord are well defined and readily traced (Brown, 1981). Not only is the anatomical organization well understood, but a number of transmitter-related substances have been identified in at least one part of the gray matter, the dorsal horn. Since DRG cells normally project to the substantia gelatinosa, homotopic grafts provide a target for these axons. Therefore, regeneration by primary afferent fibers into transplants can be demonstrated because specific markers exist for subpopulations of DRG neurons and these markers disappear from normal spinal cord following dorsal rhizotomy (Dodd and Jessell, 1985). Several of these markers such as substance P and somatostatin derive from several sources, but one of these markers is the neuropeptide calcitonin gene-related peptide (CGRP), which in the dorsal horn originates only from the dorsal roots (Gibson et al., 1984).

In several respects the potential for regeneration and plasticity of DRG neurons resembles that of other adult mammalian central neurons. For example, following axotomy in adults, the cut dorsal roots regenerate, but growth into the spinal cord is abortive (reviewed in Reier et al., 1983; Reier, 1986). Furthermore, following axotomy of adjacent dorsal roots, the synaptic terminals of spared dorsal roots show changes of morphological plasticity that resemble those described elsewhere in the CNS (Murray et al., 1987). Whether embryonic spinal cord transplants will enhance the regeneration of dorsal roots has not yet been determined. In the present study we show that regeneration occurs and that CGRP-containing axons are among those that regenerate.

Materials and methods

Sprague-Dawley rats (200 – 300 g) received transplants of E14 or E15 spinal cord at the level of the lumbar enlargement using techniques that have been described (Reier et al., 1986). A 2 – 3 mm length of one side of the spinal cord was resected, and the adjacent dorsal roots were sectioned. The transplant was then introduced into the cavity, the severed dorsal root stumps that remained attached to the DRGs of origin were juxtaposed to the

transplant, and the wound was closed. After post-operative survivals of two to nine months, dorsal roots regenerating into transplants were labeled with one of three techniques. The original wound was reopened, and the dorsal roots which entered the graft were identified. The roots were transected 5 – 6 mm from the insertion site and labeled with a solution of 10% HRP (horseradish peroxidase) and 1% WGA-HRP (wheat germ agglutinin-conjugated horseradish peroxidase) using a method similar to that described by Beattie et al. (1978). After 24 – 48 h survivals the animals were perfused, and transplants were processed for HRP visualization using 3,3′-diaminobenzidine (DAB) as the chromagen followed by cobalt chloride intensification (Adams 1981) or using the tetramethylbenzidine (TMB) protocol of Mesulam (1978).

The sciatic nerve ipsilateral to the transplant was labeled at the mid-thigh level with an intraneural injection of either 2% WGA-HRP or 0.75% cholera toxin-conjugated HRP (CT-HRP) using a modification of the method described by Harrison et al. (1984). After 48 h, the animals were perfused, and transplants were processed for the visualization of HRP according to the TMB protocol of Mesulam (1978).

Host rats were perfused, and cryostat sections cut at 15 μm through the transplants were processed on slides for the peroxidase-antiperoxidase method (Sternberger, 1979) using techniques previously described (Tessler et al., 1980). Primary antisera raised in rabbits against human CGRP were obtained from Peninsula Laboratories (Belmont, CA) and used in 1 : 16 000 dilution. Antibody specificity was verified by the absence of immunoreactive elements when primary antibody was replaced with antiserum preabsorbed with 50 μg/ml of CGRP or with CGRP alone.

Results

Areas of apposition developed between transplants and host dorsal roots and spinal cord. However, the continuity between host dorsal roots and transplants was interrupted by glial and connective tissue scarring which varied in intensity not only

between animals but also in different areas of the same interface.

Anterograde labeling (injury filling) showed that host dorsal roots had grown into ten of twelve transplants studied with this method (Fig. 1A). Predominantly small- and medium-caliber dorsal root axons were labeled. They penetrated the grafts as far as 3 mm but most remained within 2 mm of the dorsal root-transplant interface. Labeled axons did not grow through the entire rostral-caudal extent of grafts (approximately 4 mm) or pass through the transplant to enter host spinal cord in spite of the areas of fusion between transplants and host spinal cord. Within the grafts dilatations occurred along the labeled axons, and many of the fibers showed extensive spray-like arborizations and varicosities (Fig. 1B). None of the transplants studied with this technique showed retrograde cell body labeling.

Injecting WGA-HRP or CT-HRP into the sciatic nerve of seven host rats led to a reaction product in transplants consistent with transganglionic labeling of regenerated dorsal roots. The reaction product appeared primarily as clusters of fine granules and less often as individual fibers (Fig. 2). This label generally remained close to the dorsal root-transplant interface, but some fibers extended for at least 1.5 mm within the grafts. Dorsal roots did not grow across transplants into host spinal cord.

In two recipients with tracer injected into the sciatic nerve we found occasional retrogradely labeled cells within the transplants and in another two recipients we found a few retrogradely labeled cells in host spinal cord contralateral to the transplants. One specimen contained both types of labeled neurons. Cells filled within transplants were multipolar neurons measuring up to 37.5 μm

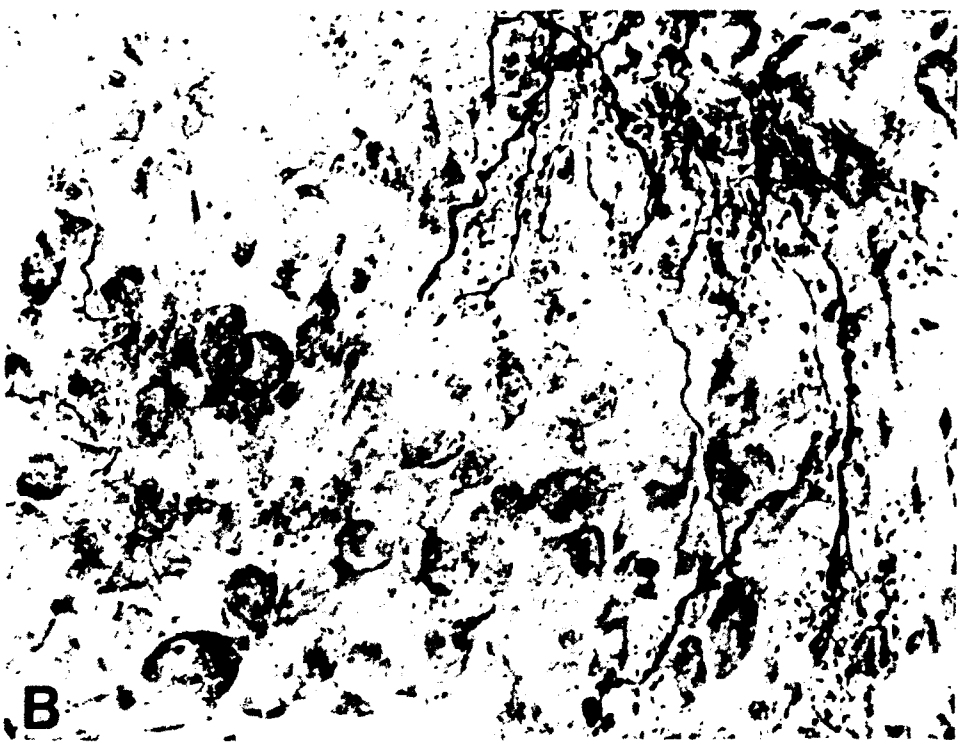

Fig. 1. Sagittal section of an embryonic spinal cord transplant in adult host spinal cord two months after transplantation. Host dorsal roots were labeled with a mixture of HRP and WGA-HRP. A. Several labeled dorsal roots are shown (at arrow) which have regenerated into the transplant. × 109. B. Higher magnification view of labeled host dorsal roots within transplant. Nomarski optics × 440. Sections counterstained with neutral red.

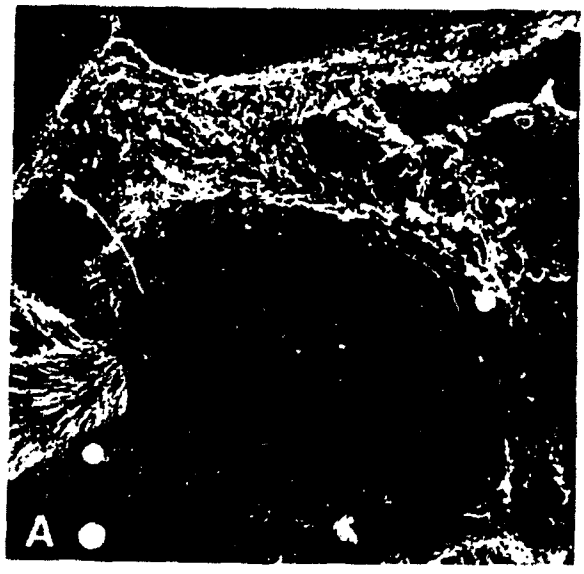

Fig. 2. Darkfield photomicrographs of transverse sections through a fetal spinal cord transplant following CT-HRP injection into ipsilateral sciatic nerve. A. This photomicrograph shows dorsal root regeneration up to, but not within the transplant. Labeling is seen in the dorsal root entry zone, but not past the host-transplant interface. × 73. B. This photomicrograph shows regeneration of host dorsal roots into the transplant. × 73.

in their longest dimension and were located in the ventral or central regions of the grafts (Fig. 2). Labeled host neurons were located ventrolateral to the central canal in laminae VIII and X of Rexed and included multipolar cells measuring up to 27.5 μm in their longest dimension.

CGRP immunoreactive fibers entered the transplants from the host dorsal root, arborized extensively, and displayed varicosities along their length (Fig. 3). They were present in the six cases studied. Most were clustered within 1 mm of the dorsal root-transplant interface, but some penetrated more deeply, and occasional processes traversed the entire dorsal-ventral extent of the transplant (1 – 2 mm). Immunoreactive fibers extended up to to 3 mm in the rostral-caudal plane. Occasional perikarya staining for CGRP was found in the grafts but only rarely in the regions of the fibers.

Discussion

Regeneration appears to depend on an interaction between the injured axon's intrinsic capacity to regrow and the environment in which the regrowth must occur. Some axons regenerate even within the generally unfavorable context of the mammalian central nervous system (CNS) (Kawaguchi et al., 1986). Other central neurons do not ordinarily regenerate, but regrowth of their axons can be promoted by grafts of peripheral (Aguayo, 1985) or fetal CNS (reviewed in Björklund and Stenevi, 1984) tissue. Exposed to the same type of graft, however, populations of neurons differ in their regenerative capacity (Aguayo, 1985), suggesting that some central neurons may be extremely limited in their ability to regrow or that the conditions necessary to elicit their growth are not provided by the usual grafts. Understandng the extent to which regeneration is limited by the metabolic features of neurons themselves (Skene and Willard, 1981; Barron 1983) and the conditions necessary to stimulate growth are fundamental to efforts to promote regeneration within the damaged CNS.

DRG neurons provide a single system in which many of these questions can be investigated. The transected peripheral processes of these neurons readily regenerate. The severed central processes also regenerate, but only within the peripheral por-

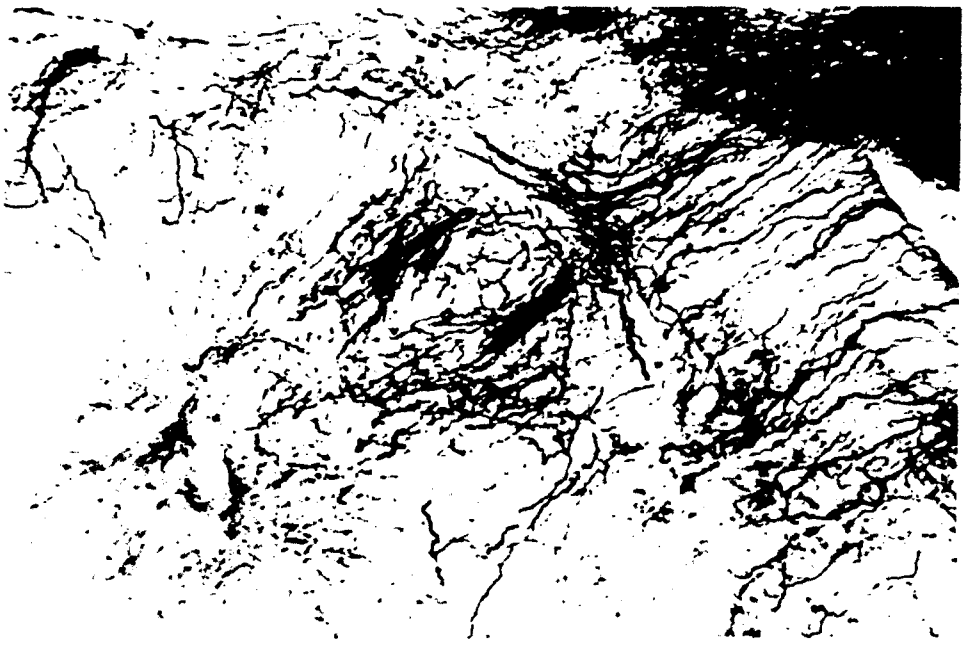

Fig. 3. Sagittal section of transplant-dorsal root interface showing CGRP-containing dorsal root afferents within the transplant × 225.

tion of the dorsal root, and not into the spinal cord (Stensaas et al., 1979; Bignami et al., 1984). Failure to enter the spinal cord therefore appears to be limited by the environment at the dorsal root entry zone rather than by the inherent inability of the axons to regrow (reviewed in Reier et al., 1983; Reier, 1986). Although the transplant may act by enhancing the regenerative vigor of the cell, thus allowing the growing axons to penetrate the dorsal root-spinal cord interface, the present results further emphasize the importance for regeneration of influences extrinsic to the neuron by showing that dorsal roots regrow into transplants of embryonic spinal cord. Tracing methods which used transganglionic transport of HRP applied to the sciatic nerve and anterograde transport of HRP applied to the dorsal roots revealed host dorsal root afferents that had penetrated into the grafts. Moreover, dorsal root afferent fibers labeled within transplants by anterograde or transganglionic transport were similar morphologically to dorsal root afferents in normal spinal cord labeled by these techniques (Beattie et al., 1978; Abrahams and Swett, 1986). These results can be interpreted in several ways: (1) that the immature environment is more permissive of regrowth than the adult and/or (2) it is more capable of enhancing the regenerative response of injured neurons.

DRG neurons have been classified into subgroups based on criteria such as size and immunocytochemical staining characteristics (summarized in Dodd and Jessell, 1985). These subgroups may differ in their capacity to regenerate or in the vigor of their response to the conducive environment or the stimulation provided by a transplant. The potential for plasticity of subclasses of uninjured DRG neurons has already been shown to vary (Mendell et al., 1987). Our results using a marker specific for a population of small- and medium-sized DRG neurons show that dorsal root cells immunoreactive for CGRP are among those which regenerate into transplants. Indeed the extent of regeneration demonstrated by neurons with this immunocytochemical label exceeds that shown by either method that depends on the axonal transport of HRP.

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Regeneration of Adult Dorsal Root Axons Into Transplants of Embryonic Spinal Cord

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ABSTRACT

Transplants of the embryonic rat spinal cord survive and differentiate in the spinal cords of adult and newborn host rats. Very little is known about the extent to which these homotopic transplants can provide an environment for regeneration of adult host axons that normally terminate in the spinal cord. We have used horseradish peroxidase injury filling and transganglionic transport methods to determine whether transected dorsal roots regenerate into fetal spinal cord tissue grafted into the spinal cords of adult rats. Additional transplants were examined for the presence of calcitonin gene-related peptidelike immunoreactivity, which in the normal dorsal horn is derived exclusively from primary afferent axons. Host animals had one side of the L4-5 spinal cord resected and replaced by a transplant of E14 or E15 spinal cord. Adjacent dorsal roots were sectioned and juxtaposed to the graft. The dorsal roots and their projections into the transplants were then labeled 2–9 months later. The tracing methods that used transport or diffusion of horseradish peroxidase demonstrated that severed host dorsal root axons had regenerated and grown into the transplants. In addition, some donor and host neurons had extended their axons into the periphery to at least the midthigh level as indicated by retrograde labeling following application of tracer to the sciatic nerve. Primary afferent axons immunoreactive for calcitonin gene-related peptide were among those that regenerated into transplants, and the projections shown by this immunocytochemical method exceeded those demonstrated by the horseradish peroxidase tracing techniques. Growth of the host dorsal roots into transplants indicates that fetal spinal cord tissue permits regeneration of adult axotomized neurons that would otherwise be aborted at the dorsal root/spinal cord junction. This transplantation model should therefore prove useful in studying the enhancement and specificity of the regrowth of axons that normally terminate in the spinal cord.

Key words: fetal spinal cord transplants, dorsal root ganglion neurons, horseradish peroxidase, anterograde transport, transganglionic transport, calcitonin gene-related peptide, immunocytochemistry

Fetal spinal cord transplants have been shown to grow and differentiate in the damaged spinal cords of newborn (Bregman and Reier, '86) and adult (Patel and Bernstein, '83; Reier et al., '86a,b) rats where such homotopic grafts can also integrate with the neuropil of the host spinal cord (Reier et al., '86b). Whereas homotopic intraspinal grafts can establish cellular bridges conducive to the regrowth of lesioned host axons in neonatal recipients (Bregman and Reier, '86; Bregman, '87a,b), the extent to which these transplants support regeneration of adult host axons is still uncertain. It also remains to be determined whether fetal spinal cord tissue can reconstruct damaged intraspinal cir-

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cuits by providing suitable targets for axons that normally terminate in the spinal cord.

The central processes of dorsal root ganglion neurons provide a particularly attractive model for studies of this sort. First, the intraspinal terminal distributions (Brown, '81; Ralston et al., '84; Sugiura et al, '86), immunocytochemical staining properties (reviewed in Dodd and Jessell, '85), and neurophysiology (reviewed in Brown and Rethelyi, '81) of primary afferent neurons have been characterized extensively. Second, the regenerative capacity of these axons has been investigated in considerable detail. After axotomy the cut central processes of adult dorsal root ganglion neurons regenerate along the degenerated dorsal root, but their elongation into the spinal cord is aborted as they approach the dorsal root entry zone (reviewed in Reier et al., '83; Reier, '86; see also Liuzzi and Lasek, '87b). In this respect, dorsal roots behave like other adult mammalian central axons (Cajal, '28). Finally, grafts of embryonic spinal cord develop differentiated regions that resemble the superficial dorsal horn of normal adult spinal cord (Reier et al., '86a,b). Since this area normally receives extensive innervation from dorsal root axons, it may, when grafted into adult spinal cord, provide a target for regenerating primary afferents.

It is presently unknown, however, whether the cut central processes of host dorsal root ganglion neurons will regenerate into transplants of embryonic spinal cord and, if so, whether they will differentiate and contain normal markers. We have therefore used neuroanatomical tracing methods to determine whether transected primary afferent axons have the capacity to regenerate into homotopic grafts placed at lumbar spinal levels in adult hosts. We have also studied whether regenerating axons of dorsal root ganglion neurons are immunoreactive for calcitonin gene-related peptide (CGRP), a specific marker for primary afferent axons that terminate in the normal dorsal horn (Gibson et al., '84). The results indicated that severed dorsal roots entered the grafts and that some of the regenerating axons were immunoreactive for CGRP.

MATERIALS AND METHODS

Surgery

Sprague-Dawley (Zivic Miller, Allison Park, PA) rats of either sex and weighing 200–300 g served as graft recipients. The animals were anesthetized with Ketamine (60 mg/kg) and Xylazine (10 mg/kg) and underwent laminectomies of the T13 and L1 vertebrae in order to expose the lumbar enlargement. The surrounding meninges were incised dorsally at the midline, and a 2–3 mm length of one side of the spinal cord was removed by sharp dissection and aspiration. Adjacent dorsal roots were sectioned and reflected back from the lesion. Segments of spinal cord approximating the length of each intraspinal cavity were removed from E14 or E15 Sprague-Dawley rat fetuses and then introduced into the lesion using procedures for donor tissue preparation and grafting previously described (Reier et al., '86a). The severed dorsal root stumps that remained attached to the dorsal root ganglia of origin were juxtaposed to the transplant. Roots were anchored by placing a strip of hydrocephalus shunt film (Durafilm, Codman Surtlef, Inc.) beneath the dura and over the lesion cavity. The dural incision was then closed with interrupted 10-0 silk sutures, and another piece of Durafilm was placed on top of the dura. The superficial wound was then closed in layers.

Labeling Methods

Dorsal root labeling. Two to 9 months after transplantation, recipients were anesthetized with Ketamine and Xylazine as described above. The original wound was reexposed, and the dorsal roots inserted into the graft site were identified. Using a method similar to that described by Beattie et al. ('78), these roots were then transected 5–6 mm from the insertion site, the spinal cord was covered with parafilm, and the cut central ends of the rootlets were placed into the end of a disposable micropipet that contained a solution of 10% HRP (horseradish peroxidase) and 1% WGA-HRP (wheat-germ agglutinin-conjugated horseradish peroxidase) for at least 1 hour. The exposure site was then covered with mineral oil and the wound closed. The animals were deeply anesthetized with Nembutal (40 mg/kg) 24–48 hours later and perfused transcardially with 100–150 ml of physiological saline followed by 300–500 ml of a solution of 2.5% glutaraldehyde-1% paraformaldehyde in 0.1 M phosphate buffer. Transplants were removed immediately, cut into segments of 4–5 mm in length, and sectioned at 40 μm thickness on a Vibratome in either a sagittal or transverse plane. The sections from each specimen were divided into two groups: one set was processed for HRP visualization using 3,3'-diaminobenzidine (DAB) as the chromagen followed by cobalt chloride intensification (Adams, '81); the other was processed according to the tetramethylbenzidine (TMB) protocol of Mesulam ('78).

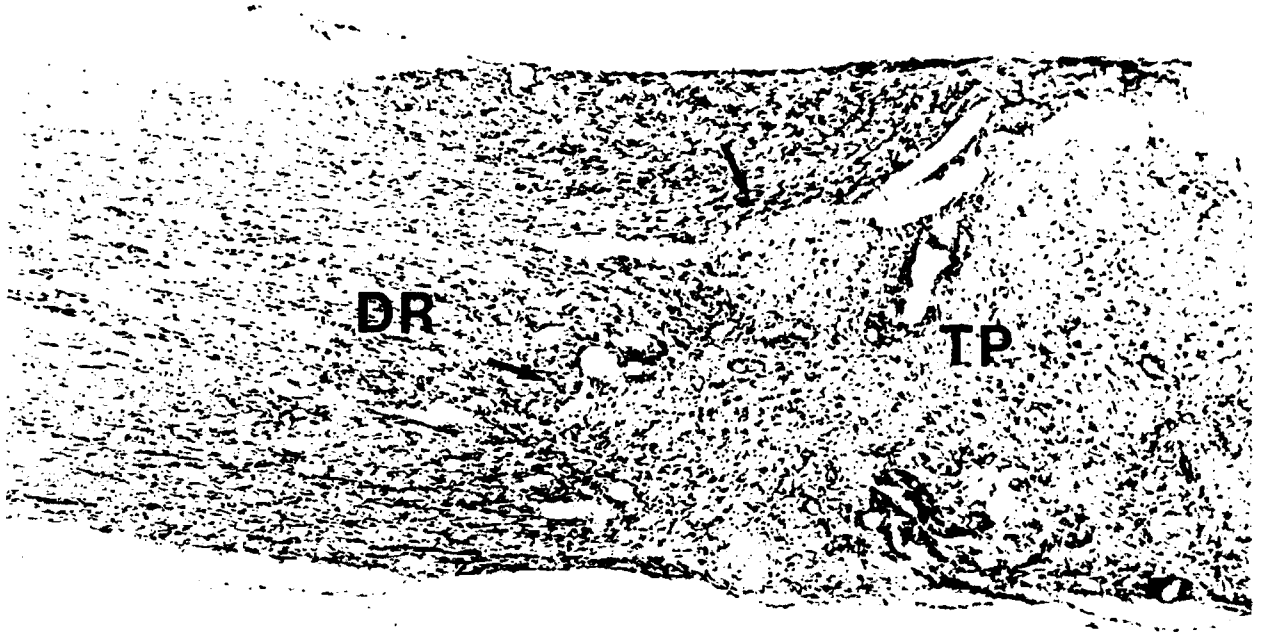
Sciatic nerve labeling. Two to 9 months after transplantation, the sciatic nerve ipsilateral to the transplant was labeled with an intraneural injection of either WGA-HRP or cholera toxin-conjugated HRP (CT-HRP). WGA-HRP was conjugated according to the method described by Mesulam ('82) using Sigma Type VI HRP and Sigma WGA. CT-HRP was conjugated following the same protocol using Sigma Type VI HRP and Sigma CT.

Using a modification of the method described by Harrison et al. ('84), the nerve was exposed at the midthigh level, freed from connective tissue, and loosely tied with a ligature of 4-0 silk. A 1–2 mm length of nerve was then crushed with a jeweler's forceps 10 mm proximal to the ligature. A total of 8–10 μl of 0.75% CT-HRP or 2% WGA-HRP was then injected over a 10-second period into the center of the nerve segment between the crush and ligature using a 10-μl Hamilton syringe with a 26-gauge needle. After withdrawing the needle, the ligature was tightened and the nerve cut distal to the ligature.

After 48 hours, the animals were deeply anesthetized with Nembutal (40 mg/kg) and perfused transcardially with an initial flush of warm saline containing 4 ml of 30% H_2O_2 per liter followed by 500 ml of chilled fixative composed of 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) over 20 minutes. The fixative was replaced by successive perfusions with 10%, 20%, and 30% solutions of sucrose in 0.1 M phosphate buffer (pH 7.4).

The transplant and the L4 and L5 dorsal root ganglia ipsilateral to the injection were removed and stored at 4°C

Fig. 1. (a) Sagittal section of interface between host dorsal root and fetal spinal cord transplant showing area of fusion after a 2-month survival period. Arrows indicate dorsal root (DR)-transplant (TP) interface. Cresyl violet stain, calibration bar = 200 μm. (b) Higher magnification of dorsal root-transplant interface in a different case showing readily identifiable transition between host dorsal root (DR) and transplant (TP). Processes within transplant are labeled with CGRP. CGRP immunocytochemistry counterstained with cresyl violet. Bar = 100 μm.



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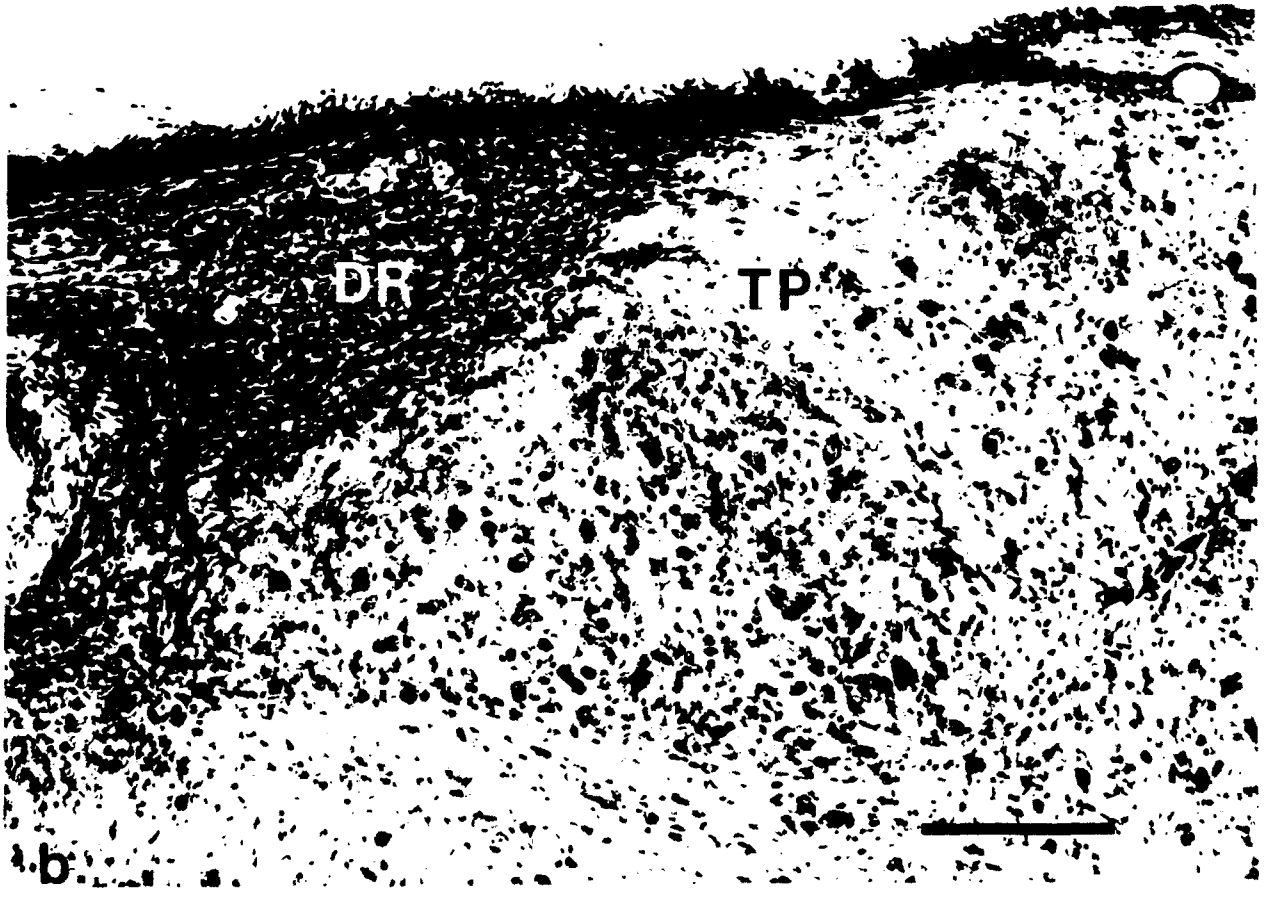


Figure 1

overnight in 30% sucrose. Transverse sections (40 μm thick) through the transplant and longitudinal sections through the dorsal root ganglia were cut on a sliding microtome and processed for the visualization of HRP according to the TMB protocol of Mesulam ('78).

CGRP immunocytochemistry. Two to 9 months after transplantation, host rats were deeply anesthetized with Nembutal (40 mg/kg) and perfused through the heart with normal saline followed by 4% paraformaldehyde or 4% paraformaldehyde-0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.4. Spinal segments that contained transplants were removed and embedded in paraffin or sectioned on a cryostat and mounted on subbed slides. Sections to be examined for CGRP were prepared for the immunocytochemical labeling procedure and for examination by light microscopy using methods that have been described previously (Sternberger, '79; Tessler et al., '80). All sections were studied for the presence and distribution of CGRP, and representative samples were drawn or photographed.

Primary antisera raised in rabbits against human CGRP were obtained from Peninsula Laboratories (Belmont, CA) and used in 1:16,000 or 1:18,000 dilution. Antibody specificity was verified by the absence of immunoreactive elements when primary antibody was replaced with antiserum preabsorbed with 50 μg/ml of CGRP or CGRP alone.

RESULTS

General histology

A total of 29 recipients with surviving transplants and satisfactorily labeled dorsal roots were examined in this study. When spinal cords were dissected at autopsy under an operating microscope, the inserted dorsal roots were found to be in continuity with the transplants, which often filled the entire lesion cavity and abutted the exposed ventral quadrants of the adjacent rostral and caudal segments of the host spinal cord.

Subsequent light microscopic examination of these specimens in Nissl-stained material confirmed that areas of fusion had developed between transplants and dorsal roots (Fig. 1a,b), as well as between the grafts and the host spinal cord (Fig. 2; see also Fig. 4b). The interfaces between transplants and the inserted host dorsal roots could easily be recognized (Fig. 1b). The zone of apposition was clearly recognizable because of the striking contrast in cell density between the numerous closely packed cells that were present within the nerve roots and the more loosely cellular fetal spinal cord graft to which the roots extended. As is characteristic of the normal dorsal root entry zone, the transition betwen the inserted dorsal roots and fetal grafts featured an interdigitation of peripheral and central tissue (Fig. 1a,b; see also Fig. 4b), which consisted of an apparent extension of narrow cellular columns into the fetal spinal cord grafts. In the majority of specimens studied, a zone of glial and connective tissue scarring could also be demonstrated that partially interrupted the continuity between dorsal root and transplant. Such scarring was more pronounced in some animals than in others and also varied in intensity in different areas of the same graft. The grafts extended for approximately 4 mm rostracaudally.

Introduction of tracer into host dorsal roots

Anterograde labeling (i.e., injury filling) of the host dorsal roots with HRP/WGA-HRP demonstrated that primary afferent fibers had grown into fetal spinal cord grafts in 10 of the 12 hosts examined with this labeling procedure. There was, however, considerable interanimal variability in the relative density of the labeling: in two transplants, dorsal root ingrowth was particularly robust (Fig. 3), whereas penetration into the graft was very modest in three others. An intermediate extent of ingrowth was observed in the remaining five animals. A dense scar completely encapsulated the inserted dorsal roots in the two animals with well-labeled axons but without innervation of the transplants.

Predominantly small- and medium-caliber HRP-filled axons entered the grafts, and these appeared to have grown through areas of the dorsal root-transplant interface that lacked a dense scar or where blood vessels had penetrated the grafts (Fig. 2a). Dilatations occurred along the course of the labeled axons, and many of the fibers showed extensive spraylike arborizations and varicosities within the transplants (Fig. 2b,c), particularly in areas that contained aggregates of neuronal cell bodies (Fig. 2c). Only elongated unbranched profiles were observed in regions of the transplants in which neuronal cell bodies were sparse.

Some axons penetrated as far as 3 mm, but most of the labeled dorsal root fibers in the grafts were located within 2 mm of the root/graft junction. None of the labeled axons grew through the entire rostrocaudal length of the grafts, which extended for approximately 4 mm. Consequently no HRP-labeled axons advanced into the neighboring host spinal cord even though donor tissue was often fused both rostrally and caudally with lesioned surfaces of the host ventral gray matter.

Although tracer was applied to host dorsal roots within 5–6 mm of the grafts, no cell body labeling was observed in any of the transplants.

CGRP immunocytochemistry

CGRP staining was present in the nine grafts studied although the amount of ingrowth varied among transplants and in different areas of the same transplant. Primary afferent axons containing CGRP coursed longitudinally within dorsal roots that had been inserted into the fetal spinal cord grafts, and many of these axons could be traced extending into the donor neuropil (Fig. 4a,b). Some of these axons appeared to enter the grafts via longitudinally oriented fingers of peripheral tissue at the newly established PNS-CNS transition zone (Fig. 4a,b). CGRP-immunoreactive processes arborized extensively within the grafts and showed varicosities along their length (Fig. 4a,b). The densest staining was found within 1 mm of the dorsal root-transplant interface. Some fibers penetrated more deeply (Fig. 4a), and occasional processes extended across the entire dorsal-ventral extent of the transplant (1–2 mm) and could be seen near the host spinal cord-graft interface. In the rostral-caudal plane immunoreactive fibers were traced for up to 3 mm. Parts of the transplant more than 3 mm from an ingrowing dorsal root as well as regions close to a dorsal root that failed to penetrate the host-transplant in-

Fig. 2. Sagittal section through area of embryonic spinal cord graft 2 months after transplantation. Host dorsal roots were labeled with a mixture of HRP and WGA-HRP. (a) Labeled dorsal roots (arrow) are shown that have regenerated into the graft (TP), but not into host spinal cord (H). Area indicated by asterisk is shown at greater magnification in b. Bar = 100 μm. (b) Labeled regenerated axons cross the dorsal root entry zone and arborize within the transplant. Bar = 100 μm. (c) Higher magnification showing fine detail of the arborization of another labeled axon within the transplant. Nomarski optics, bar = 100 μm.

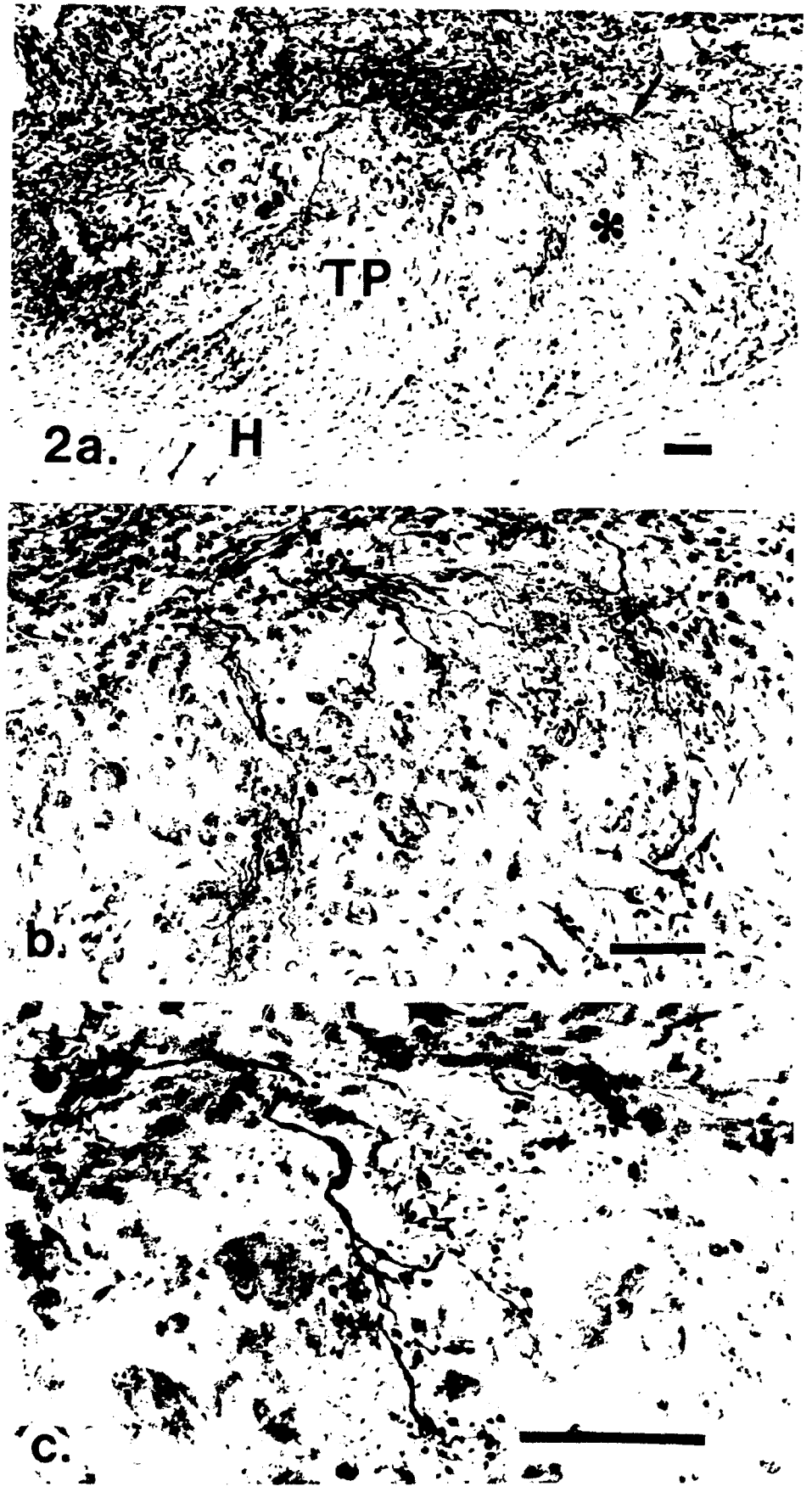


Figure 2

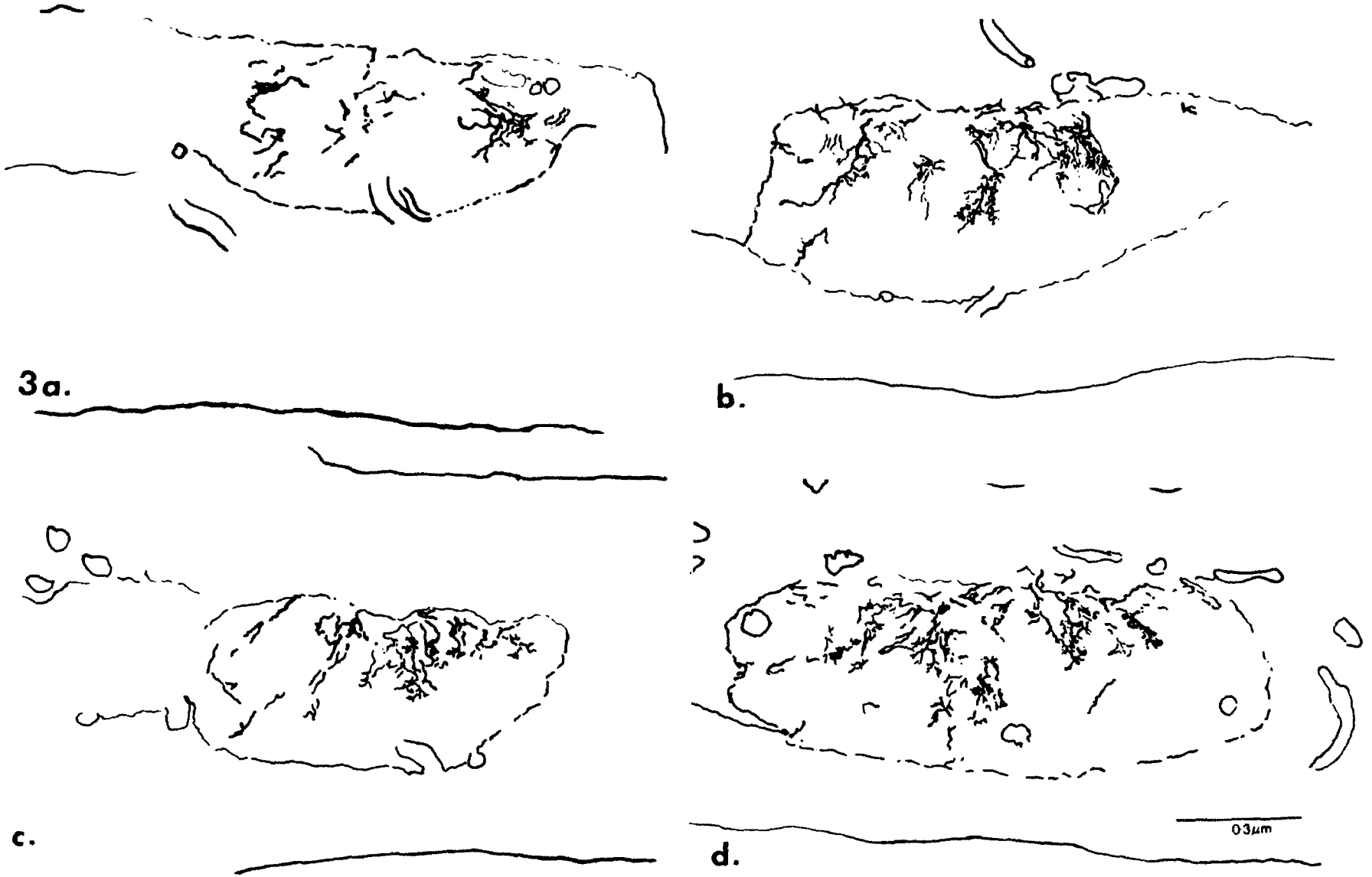
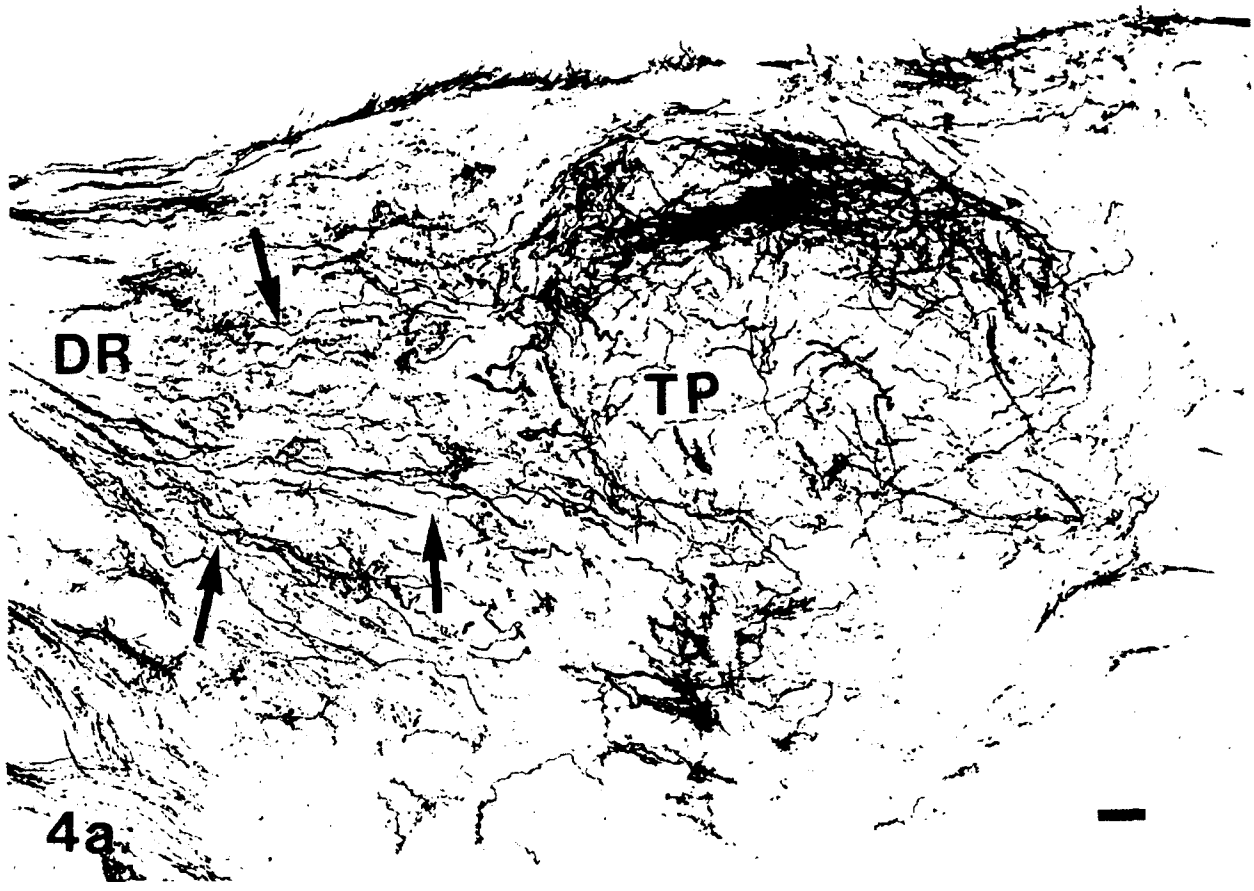


Fig. 3. Four representative camera lucida drawings showing the general distribution and density of HRP-anterogradely filled dorsal root fibers and arborizations in a spinal cord transplant. The distances between sections are as follows: a and b = 40 μm; b and c = 120 μm; c and d = 80 μm. The scale bar is shown in (d). Note that most of the arborizations occur just beyond the dorsal root/graft interface.



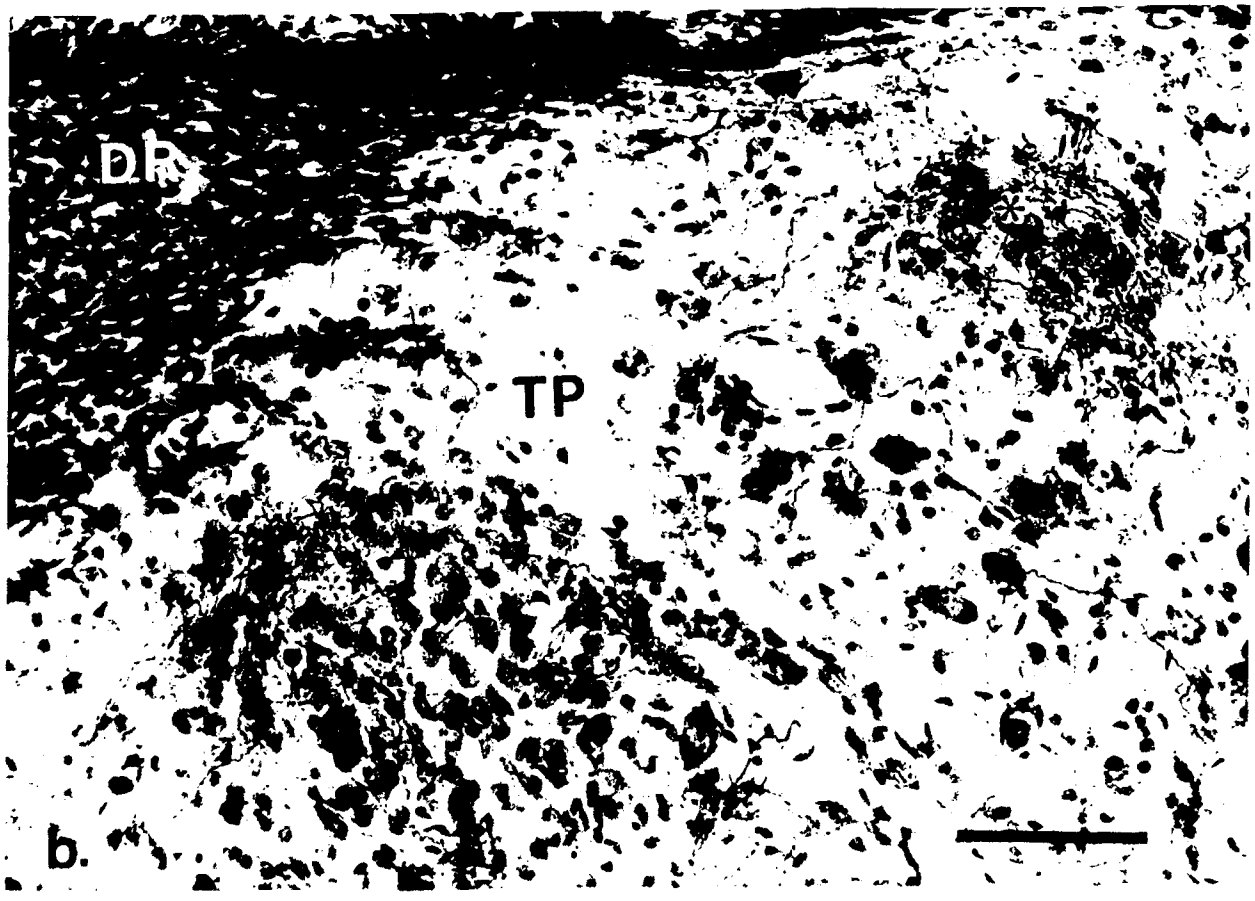


Fig. 4. (a) CGRP staining in embryonic spinal cord transplant showing immunoreactive axons in host dorsal root (DR) juxtaposed to transplant (TP) and in some cases passing between host and graft (arrows). 2 month survival. Bar = 100 μm. (b) Higher power view of same transplant shown in Figure 4b. CGRP immunoreactive axons are shown crossing between host dorsal root (DR) and transplant (TP) and branching in densely cellular areas of transplant (*). Bar = 100 μm.

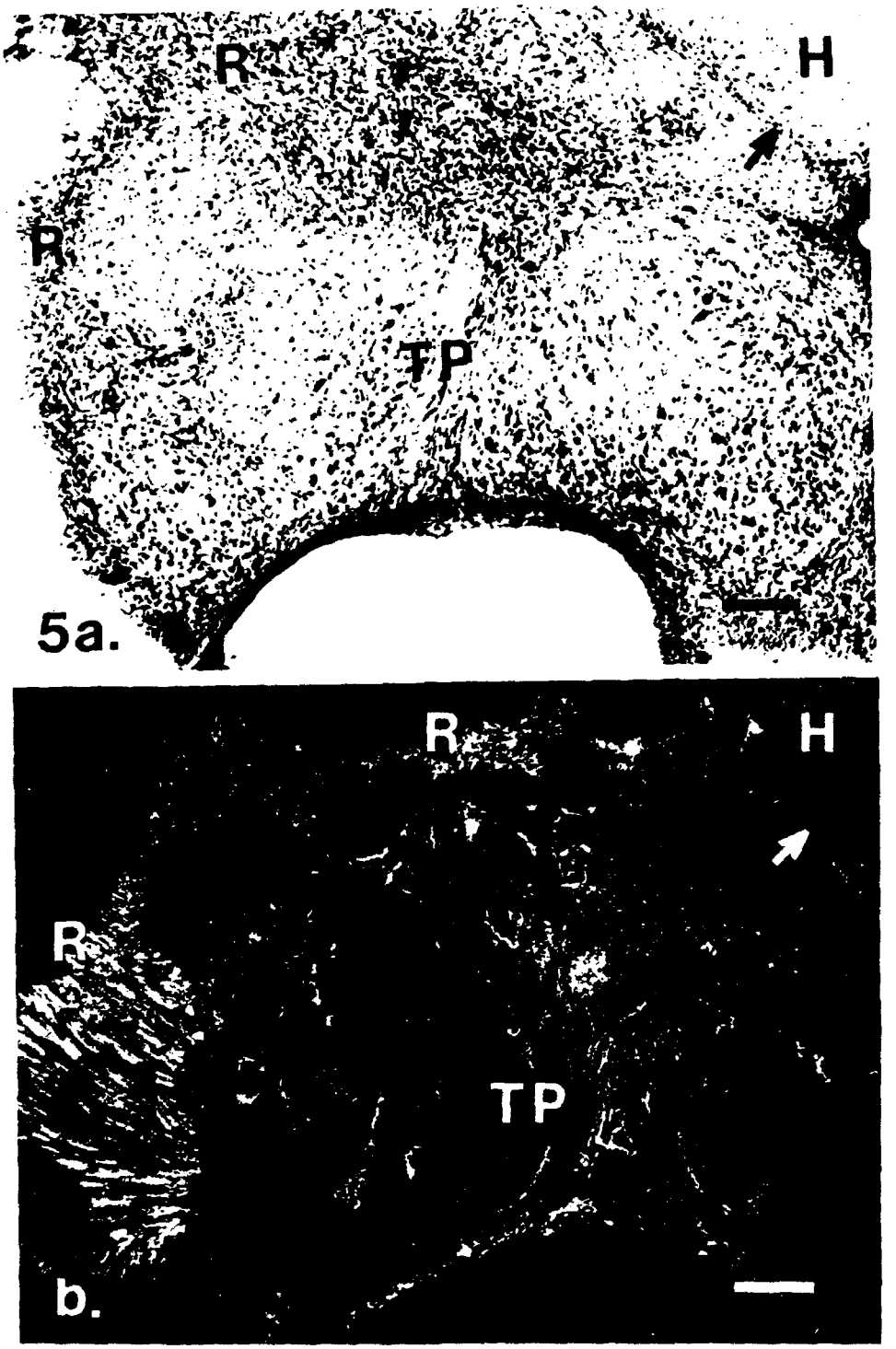
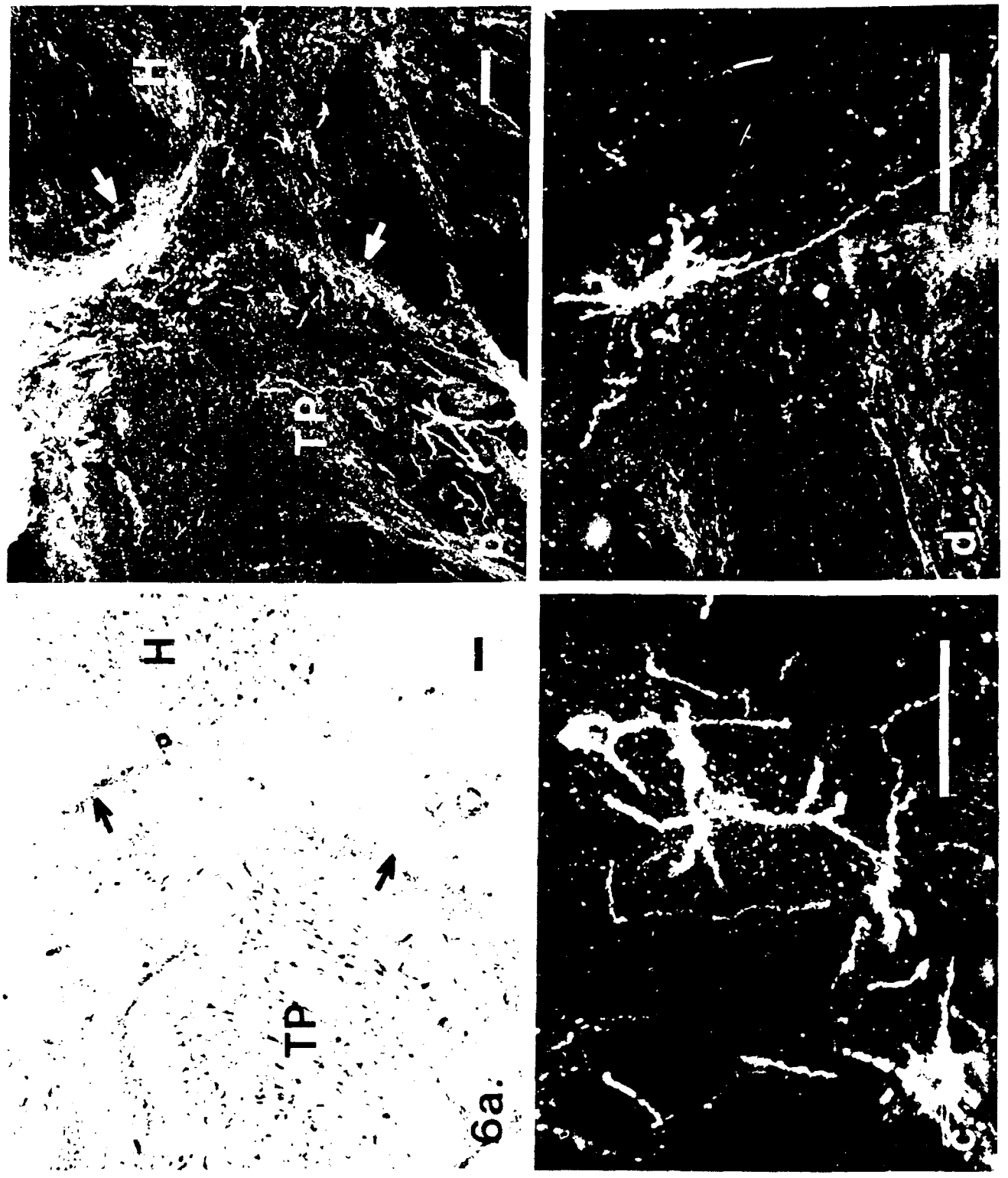


Fig. 5. Transverse section through an area of embryonic spinal cord graft 3 months after transplantation into host spinal cord. Host sciatic nerve was injected with WGA-HRP. (a) Brightfield photomicrograph of a cresyl violet stained section showing relationship of fetal transplant (TP) to host dorsal roots (R) and host spinal cord (H). Arrow indicates host transplant interface. Bar = 100 μm. (b) Photomicrograph of an adjacent section using darkfield illumination to show labeled dorsal roots (R) and labeled fibers throughout the graft (TP). Arrow indicates transplant interface with host (H) spinal cord. Bar = 100 μm.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terface had few or no processes positive for CGRP-like staining. Occasional perikarya staining for CGRP were found in the grafts but rarely in the regions of the fibers.

Injection of tracer into host sciatic nerve

Injections of WGA-HRP or CT-HRP into the sciatic nerves of seven graft recipients led to extensive labeling of the inserted dorsal roots prior to their entry into the transplants and to a granular and/or fibrous staining product in the neuropil of the transplants. In these preparations no labeled dorsal root fibers extended across transplants into adjacent host gray matter.

Transganglionic labeling of regenerated dorsal roots appeared primarily as clusters of fine granules and less often as individual fibers (Figs. 5, 6). Such labeling was densest in zones close to the dorsal roots-graft apposition sites, but some longitudinal axonal profiles extended for at least 1.5 mm within the depth of the grafts (Fig. 5).

In contrast to the results obtained with the injury filling technique, injection of WGA-HRP and CT-HRP into the sciatic nerve also demonstrated retrograde neuronal labeling within grafts of two of the seven recipients (Fig. 6). These cells were multipolar neurons that measured from 22.5–37.5 μm in longest diameter with their perikarya positioned either in the ventral or more central regions of the transplants. In some cases the processes of these cells branched extensively within the grafts.

In one animal with WGA-HRP and one animal with CT-HRP injected into the sciatic nerve, a few retrogradely labeled neurons were observed in the host gray matter adjacent to the transplant (Fig. 6). The animal injected with CT-HRP, which demonstrated labeled neurons in host spinal cord, also had labeled neurons within the transplant. Labeled host neurons were located ventrolateral to the central canal in laminae VIII and X of Rexed and included multipolar neurons measuring 22.5–27.5 μm in longest diameter.

DISCUSSION

Following axotomy, the cut central processes of adult mammalian dorsal root ganglion neurons regenerate vigorously, but only in the dorsal root (Stensaas et al., '79; Bignami et al., '84). As the regenerating fibers approach the dorsal root entry zone of the spinal cord, their elongation ceases (Stensaas et al., '79; Bignami et al., '84), and few if any regenerating axons reenter the spinal cord. This failure to advance more centrally has been attributed to the inhibitory effects of astrocytes at the PCS-CNS transition zone rather than to an intrinsic inability of these axons to regrow (reviewed in Reier et al., '83; Reier, '86; Liuzzi and Lasek, '87a).

The results of the present study, which demonstrate regeneration of adult host dorsal roots into transplants of embryonic spinal cord, further emphasize the importance of extrinsic influences on the regenerative behavior of mature CNS axons. Apparently these axons have a greater capacity for growth within the environment of fetal spinal cord tissue than they do in mature spinal cord. That immature CNS tissue enhances the elongation of these axons has also been recently illustrated in the neonatal rat spinal cord (Carlstedt et al., '87). In that study primary afferent axons were observed to regenerate into the spinal cord after crush injury in the first postnatal week, but not thereafter. The present experiments demonstrate that a sufficiently favorable environment consisting of fetal spinal cord tissue can enhance the regenerative capacity of mature spinal sensory neurons even when the roots have been transected rather than crushed.

Although dorsal root axons were able to penetrate fetal graft tissue, the density of ingrowth varied from one animal to another. Our observations suggest that the most extensive regeneration occurred when integration between donor and host spinal cord was greatest. Partial misalignment of damaged roots with intraspinal transplants and more extensive scar formation at the dorsal root-graft interface could account at least in part for those cases in which ingrowth was modest. Like the regeneration, these factors were also variable. Scarring, for example, occurred at graft sites in all recipients, but the distribution and density of the scar varied among animals. The contribution of each of these extrinsic factors to the prevention of regeneration requires elucidation.

Our conclusion that labeled processes within transplants originated from regenerated host dorsal root ganglion neurons rather than from labeled spinal neurons is strongly supported by the results obtained with the anterograde labeling (injury filling) procedure. This technique demonstrated labeled processes in the grafts, and these displayed the morphological appearance of axons in the absence of any retrogradely labeled perikarya. Therefore the labeled processes cannot be attributed either to collateral axons or to the dendrites of donor neurons that had entered the dorsal roots or to labeled neuritic processes of extensively filled donor neurons. In the two transplants in which retrogradely labeled neurons were demonstrated after injection of HRP into the sciatic nerve, however, it was difficult to distinguish unequivocally individual primary afferent fibers from the filled neuritic processes of the retrogradely labeled neurons.

In addition to the results obtained with injury filling, complementary evidence for dorsal root regeneration into grafts was derived from CGRP and transganglionic labeling methods. Transganglionic labeling has been used extensively for charting the distribution of primary afferents (Grant et al., '79; Mesulam and Brushart, '79; Swett and Woolf, '85; Robertson and Grant, '85; Abrahams and Swett, 86). With this procedure labeled fibers in grafts resembled those seen in normal cord. Furthermore, these axons were found in transplants that lacked retrograde neuronal labeling and in regions of transplants distant from those that did contain labeled neurons. Numerous fibers within transplants had the morphological appearance of HRP-labeled axons and were also immunoreactive for the neuropeptide CGRP. In normal dorsal horn CGRP is derived exclusively from dorsal root ganglion neurons (Gibson et al., '84), and even after the administration of colchicine, CGRP-containing perikarya have not been observed in the dorsal horn but only in large neurons within the ventral horn, which are probably motoneurons (Skofitsch and Jacobowtiz, '85). In many cases CGRP processes were labeled in the dorsal root and could be seen to enter the transplant from the dorsal root where they were most densely clustered in the region of the dorsal root-transplant interface. These results using an immunocytochemical method indicate regeneration of the transected adult dorsal root and are consistent with those obtained using HRP tracing. The number of primary afferent axons demonstrated within transplants by the CGRP-labeling method is greater and their ingrowth more extensive than those labeled with HRP. It is likely, therefore, that methods that depend on the transport or diffusion of HRP underestimate the extent of host dorsal

root ingrowth. The explanation for this apparent underestimation of axon ingrowth by HRP tracing methods is uncertain. One possible explanation for the limited labeling seen with transganglionic transport is that only a portion of the dorsal root ganglion neurons whose peripheral processes are in the sciatic nerve have regenerated. Similarly only a small portion of the regenerated dorsal roots may be exposed to HRP when tracer is applied directly to the dorsal roots in the procedure used for injury filling. Alternatively, the restricted labeling following sciatic nerve injection may be due to impaired transport of HRP at any point between the peripheral nerve injection site and dorsal root projections into the transplants, and the limited labeling after dorsal root application may be due to limited diffusion of HRP in recently regenerated axons. In contrast, approximately 50% of dorsal root axons originate in dorsal root ganglion neurons that are immunoreactive for CGRP (Gibson et al., '84).

Dorsal root axons entered the fetal spinal cord grafts and grew for up to 3 mm. As seen with CGRP immunostaining, the extent to which they ramified and arborized within the grafts in many instances appeared comparable to that seen in the normal spinal cord. Once having entered the grafts, however, the distance of axonal ingrowth was limited and resembled that seen when Schwann cell bridges are used as grafts (Aguayo, '85). Elongation of these axons into fetal tissue may have been restricted by the progressive maturation of the neuropil and glia within the transplants; initially, immature glial cells in the transplants may be especially permissive to axonal elongation and the ability of astrocytes to support regeneration may decline as they age, as has been demonstrated in the normal animal (Smith et al., '86). Another possibility is that dorsal roots formed synapses with donor neurons (Bernstein and Bernstein, '67) or axo-glial terminals on donor astrocytes (Liuzzi and Lasek, '87a), thus providing a signal for elongation to cease. These possibilities are not mutually exclusive. However, our observation that dorsal roots formed frequent arborizations and varicosities near clusters of neuronal cell bodies favors the establishment of synapses on neurons as the responsible mechanism.

This pattern of growth of the dorsal roots in the transplant resembles that which we have observed when intraspinal fiber systems are presented with a fetal spinal cord graft (unpublished observations). Preliminary studies using immunocytochemical techniques, for example, have shown that descending host serotonergic axons also grow only a limited distance after traversing the host-fetal spinal cord graft interface. The observation that scar formation by glia and connective tissue appears to be an important factor in determining the extent of host dorsal root-graft axonal projections also parallels results that have been obtained in initial studies of the axonal projections formed between grafts and intraspinal neurons (Reier et al., '86b). Thus, although embryonic spinal cord transplants offer the potential for enhancing the ability of adult central nervous system axons to regenerate, the conditions under which growth can be maximized remain to be defined.

The presence of retrogradely labeled cells after sciatic nerve labeling is of interest because it indicates that some of the graft neurons had sent axons down the sciatic nerve to at least the site of labeling in the midthigh. This axon outgrowth represents elongation of processes from cells originating in the transplants. It is unlikely that these cells were labeled by transsynaptic, rather than retrograde, transport since the animals survived for only 48 hours after the application of tracer and were labeled not only by WGA HRP but also by CT-HRP, which does not undergo transneuronal transfer in the primary afferent system (Robertson and Grant, '85). Previous studies have shown that PNS tissue introduced into the CNS can stimulate axonal outgrowth from a variety of neuronal populations (reviewed in Aguayo, '85). It has also been shown that axonal elongation from fetal CNS tissue can be promoted when transplants are combined with a PNS graft (Aguayo et al., '84) or when embryonic grafts are introduced directly into the sciatic nerve (Richardson and Issa, '84; Bernstein and Tang, '84). We presently have no direct information concerning the route taken by donor-derived axons into the periphery. However, the labeling of transplant neurons by retrograde transport after applying tracer to the sciatic nerve, but not after injury filling, suggests that axons from these neurons had emerged via the ventral rather than the dorsal roots. Since grafts often showed excellent fusion with preserved ventral gray matter, it is conceivable that axons from donor cells within the transplants extended a short distance into the host spinal cord and then into the ventral rootlets. This would be consistent with the elongation exhibited by injured adult motor neurons, which have been shown to regenerate through white matter into nearby ventral rootlets after axotomy within the substance of the spinal cord (Risling et al., '83). Alternatively, it is possible that damaged ventral roots within the lesion cavity became closely approximated with the fetal spinal cord grafts and may have become directly innervated by donor neurons. Previous studies have shown, for example, that denervated ventral roots can attract sprouting axons after avulsion of these roots and reimplantation into the adult rat spinal cord (Carlstedt et al., '86).

Axons from the retrogradely labeled host neurons that we observed in the contralateral hemisegment of the host spinal cord may have followed a similar course into the periphery as did the donor neurons. These neurons were located primarily in lamina VIII. Neurons in this region send axons (Carstens and Trevino, '78; Willis et al., '79) and dendrites (Light and Metz, '78) across the midline, and it is likely that these processes would have been injured when spinal tissue was removed prior to insertion of the transplant. Regeneration may then have proceeded into and across the transplant, into the ventral root, and then along the sciatic nerve.

The results of the present study therefore demonstrate that transplants permit regeneration of transected axons of adult neurons whose regrowth into the mature spinal cord is otherwise abortive and at least some of these neurons retain the ability to synthesize and transport a peptide thought to function as a neurotransmitter or neuromodulator in the normal dorsal horn (Rosenfeld et al., '83; Gibson et al., '84).

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APPENDIX 16

Submitted

RESTORATION OF SUBSTANCE P AND CALCITONIN GENE-RELATED PEPTIDE IN DORSAL ROOT GANGLIA AND DORSAL HORN AFTER NEONATAL SCIATIC NERVE LESION.

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ABSTRACT

Dorsal root ganglion (DRG) neurons decrease SP synthesis after peripheral nerve lesions. Levels in the dorsal horn also decline but return to normal if regeneration is successful. In adults, when regeneration is prevented, recovery of SP in the dorsal horn is slow and incomplete, whereas, in newborns, recovery is rapid and complete even though retrograde cell death of DRG neurons is greater than in adults. We have examined the mechanisms that might account for the rapid and complete recovery of SP in the dorsal horn after peripheral nerve injury in newborns. We also examined changes in CGRP levels, which are known to decrease in the dorsal horn after nerve lesions in adults. Unlike SP, CGRP in the dorsal horn derives solely from DRG neurons. Neonatal rats received right sciatic nerve transection, and the nerves were ligated to prevent regeneration. Peptides were compared in the L4 and L5 DRG and spinal cord segments of normal rats and in rats surviving 6 days to 4 months after sciatic nerve section.

Sciatic nerve section/ligation in newborns produced 50% neuron death in L4 and L5 DRGs, but immunocytochemical methods showed that both SP-IR and CGRP-IR recovered in dorsal horn to levels indistinguishable from the control side. SP-IR recovery was not an artefact due to shrinkage because radioimmunoimmunoassay (RIA) confirmed identical amounts ipsilateral and contralateral to the lesion. The amount of SP in ipsilateral L4 and L5 DRGs was ~50% less than in control DRGs. β–PPT-mRNA hybridization and SP-IR were observed mostly in small neurons (mean size=444μm^2). The population of α-CGRP-mRNA hybridized and CGRP-IR neurons was more heterogeneous and included small, medium, and a few large cells (mean=548μm^2). The percentage of DRG neurons that contained SP (~25%) or CGRP (~50%) was the same in normal newborn and adult rats. Because neonatal nerve lesion caused a 50% loss of SP- and CGRP-IR neurons, neither selective cell survival nor

change in neuron phenotype contributed to recovery in the dorsal horn. In both L4 and L5 DRGs ipsilateral to the lesion, the surviving neurons exhibited the same level of hybridized β–PPT-mRNA and α-CGRP-mRNA as the intact neurons of control DRGs. Because neither the constitutive level of expression of the genes nor the levels of the peptides increased above those observed in intact DRG neurons, these mechanisms were also not responsible. DRG neurons axotomized neonatally do, however, contribute to recovery because recutting the sciatic nerve 2 months later induced a new partial depletion of SP and CGRP in the dorsal horn. In addition recovery was due in part to sprouting by neurons in intact DRGs rostral and caudal to L4 and L5 because sectioning these dorsal roots also depleted recovered SP and CGRP. The contribution to recovery of metabolic alterations in the neonatally injured neurons, including increased peptide transport or prolonged peptide half-life, remains to be determined.

INTRODUCTION

Dorsal root ganglion (DRG) neurons provide an accessible model in which to compare the consequences of axotomy in adult and neonate and to study the mechanisms underlying the differences in anatomical and physiological reorganization that follow in the CNS in each case. After injury to the peripheral processes by nerve section (reviewed in Aldskogius et al., '85), arthritic inflammation (reviewed in Dubner and Ruda, '92), or capsaicin administration (Nagy and van der Kooy, '83; Shortland et al., '90), some cells die and modifications occur in the surviving axotomized DRG neurons, in their central processes within the spinal cord, and also trans-synaptically in neurons at spinal and supraspinal levels (Garraghty and Kaas, '91).

Anatomical plasticity is commonly considered to be more robust after injury in neonates than after the same lesion made in adults (Bregman and Goldberger, '83; Leonard and Goldberger, '87a,b; Shortland et al., '90; Hammond and Ruda, '91; Wang et al., '91). Neonatal sciatic nerve lesions, for example, elicit much more extensive sprouting of saphenous nerve afferents within the spinal cord than sciatic nerve section in the adult (Fitzgerald, '85; Fitzgerald and Vrbova, '85; Fitzgerald et al., '90). Neonatal peripheral nerve lesions also result in the death of approximately twice the number of DRG cells as die after the same lesion in adult, indicating the greater vulnerability to axotomy of immature DRG neurons (Bondok and Sansone, '84; Yip et al., '84; Himes and Tessler, '89). The modifications in the metabolic activity of surviving DRG neurons (Perry and Wilson, '81; Basi et al., '87) include decreased synthesis of some neuropeptides, such as substance P (SP) (Jessell et al., '79; Barbut et al., '81; Tessler et al., '85; Nielsch et al., '87; Nielsch and Kean, '89; Himes et Tessler, '89), calcitonin-gene-related peptide (CGRP; Noguchi et al., '90), cholecystokinin (Shehab and Atkinson, '86; Klein et al., '91) and somatostatin (McGregor et al., '84), and increased synthesis of other peptides, e.g. vasoactive intestinal peptide, neuropeptide-Y and galanin (McGregor et

al., '84; Shehab and Atkinson, '86; Shehab et al., '86; Hökfelt et al., '87; Nielsch and Kean, '89; Villar et al., '89; Noguchi et al., '89; Villar et al., '91; Wakisaka et al., '91). Recovery of peptide levels in the dorsal horn after peripheral lesions in adults is slow and incomplete (Himes and Tessler, '89) unless successful regeneration re-establishes peripheral connections (Nielsch and Keen, '89; Henken et al., '90; Knyihar-Csillik et al., '90). The DRG neurons that survive axotomy in the neonate, however, rapidly recover the capacity to synthesize and transport peptides, e.g. SP, to the dorsal horn, and this occurs whether the peripheral process regenerates or not. Fewer surviving DRG cells, therefore, appear to supply greater amounts of peptides to the dorsal horn.

The requirement for successful peripheral regeneration for recovery of SP levels in the DRG and dorsal horn in the adult is thought to indicate that synthesis of SP is regulated by NGF (Fitzgerald et al., '85; Lindsay and Harmar, '89; Wong and Oblinger, '91) provided by retrograde transport from the peripheral target (Stoeckel et al., '75; Goedert et al., '81). Target-derived neurotrophic factors seem to be less important in the neonate, because SP recovers fully in the dorsal horn even if regeneration of the cut sciatic nerve is prevented (Himes and Tessler, '89).

The goal of the present studies was to examine possible mechanisms that might account for the rapid and complete recovery of SP in the dorsal horn after neonatal lesion in the absence of regeneration. We tested the following hypotheses: **(1)** Selective survival of peptidergic DRG neurons accounts for the recovery; **2)** Increased peptide synthesis by surviving axotomized DRG neurons because of increased SP gene expression or translation accounts for the recovery; **(3)** Restoration of staining density is an artefact of lesion induced shrinkage; **(4)** Sprouting from adjacent intact DRGs accounts for the recovery.

We also compared changes in CGRP levels in DRGs and dorsal horn. CGRP is contained in many of the same small and medium size DRG neurons that contain SP, but about 50% of DRG neurons contain CGRP whereas only 20% contain SP (Hokfelt et al.,

'78; Price, '85; Ju et al., '87; McCarthy and Lawson, '90). CGRP is similar to SP with respect to its release and distribution in the dorsal horn, and its synthesis is also regulated by NGF (Lindsay and Harmar, '89) provided by retrograde transport from peripheral targets (Inaishi et al., '92). Therefore after axotomy in the adult, access to NGF is limited and CGRP is decreased in both DRG and dorsal horn (Noguchi et al., '90; Inaishi et al., '92). Because CGRP (Gibson et al., '84; Chung et al., '88), unlike SP (Barber et al., '79), is supplied entirely to the dorsal horn by DRGs, changes of its levels occurring in the dorsal horn are specifically attributable to DRG neurons. We therefore compared the effects of neonatal sciatic nerve lesion on CGRP levels and its mRNA with those of SP.

Some of the results have been reported in preliminary form (Nothias et al. '91, Nothias et al.'92).

MATERIALS AND METHODS

Animals and surgical procedures

Sprague-Dawley rats (Zivic Miller, Allison Park, PA) were studied. New-borns were of either sex (24 hours after birth, P1; n=30), adults (225-250 g; n=6) were females. The neonates were anesthetized with hypothermia, and the adults with an i.p. injection of a mixture of ketamine (20mg/Kg), xylazine (2mg/Kg), and acepromazine (0.15mg/Kg). The right sciatic nerve was exposed and sectioned in the middle one-third of the thigh. In order to prevent regeneration, a length of the nerve was resected, and the proximal stump was ligated. Rats survived for 2 months or underwent a second surgical procedure. To determine whether axotomized DRG neurons contributed to recovery of SP- and CGRP-IR in L4 and L5 dorsal horn, 4 rats operated on the day of birth had the right sciatic nerve recut proximal to the initial lesion 2-6 months later and were allowed to survive for 15 more days. To determine whether neurons in the intact DRGs (rostral and caudal to the L4 and L5 DRGs) contributed to recovery in the L4 and L5 dorsal horns, 2 neonatal operates had dorsal roots L2, L3, L6 and S1 cut, leaving dorsal roots L4 and L5 intact. The rhizotomy was performed 3 months after neonatal sciatic nerve lesion, and rats sacrificed after 2 additional weeks.

Immunohistochemistry

Rats were anesthetized by an overdose of sodium pentobarbital (64.8 mg/kg), then perfused intracardially with saline (0.9%) followed by 4% paraformaldehyde in 0.2 M phosphate buffer (PB, pH 7.2). The L4 and L5 spinal cord segments and right and left DRG were dissected, removed, cryoprotected overnight with 30% sucrose in PB, and stored at -70 ^{0}C. Transverse sections (12 μm-thickness) were cut on a cryostat and mounted alternately on gelatin (Sigma) and polylysine (Sigma) -coated slides. Adjacent sections were incubated with primary antiserum directed against either CGRP or SP (rabbit anti-serum, Peninsula Labs, Belmont, CA) diluted at 1: 5000 in 0.2 M PB with 0.9% saline (PBS, pH 7.2) for 24 hours at room temperature, then with biotinylated goat anti-rabbit IgG and with avidin-biotinylated horseradish peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA) as specified by the manufacturer. The peroxidase activity was visualized with 0.05% diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide in 0.05 M Tris buffer. Adjacent sections were stained with cresyl-violet for counts used to estimate the total number of neurons per ganglion.

RNA probe synthesis and *in situ* hybridization histochemistry

The methods used for synthesis of ^{35}S-radiolabeled RNA probes and *in situ* hybridization histochemistry have been previously described in detail (Chesselet et al., '87).

L4 and L5 DRGs were quickly removed from deeply anesthetized rats and kept frozen until sectioning. Sections (12 μm-thickness) were cut on a cryostat, thaw-mounted alternately on gelatin- (Sigma) and polylysine (Sigma) -coated slides, and stored at -70 ^{0}C.

Two cDNAs were used as templates: for β-preprotachykinin (PPT), cHSp11 isolated from human brain library (fragment exons 2 to 6, 350-base pair, generously provided by Dr. H.U. Affolter), and for rat α-CGRP (cDN-RsaI fragment exons 2-6, 500-

base pair, generously provided by Dr. S. Leff and M.G. Rosenfeld). The β-PPT and α-CGRP cDNA were inserted respectively into the transcription pSP65 and pSP64 vectors. β-PPT- and α-CGRP-cDNA, both inserted with SP6 promotor, were restricted appropriately and transcribed by SP6-RNA polymerase according to the manufacturer's specification using 2.5 μM ^{35}S-UTP (100Ci/mmol, New England Nuclear) and 10 μM unlabelled UTP, with all other nucleotides unlabeled and present in excess. Both probes have been previously characterized by Northern blot analysis (Bonner et al., '87; Rosenfeld et al., '92).

Mounted sections were quickly brought to room temperature under a cool stream of air and fixed in 3% paraformaldehyde, rinsed in buffer 2x SSC (standard saline citrate, 1x SSC contains 0.15 M NaCl/0.015 M sodium citrate), acetylated, treated with Tris-glycine, rinsed in buffer 2x SSC, dehydrated in graded ethanol, and air dried. Twenty to 30 μl aliquots of hybridization mixture (40% formamide, 10% dextran sulfate, 4x SSC, 10 mM dithiothreitol, 1mg/ml sheared salmon sperm DNA, 1 mg/ml *E. coli* tRNA, and 1x Denhardt's solution (0,02% Ficoll, 0,02% polyvinyl pyrolidone, 10mg/ml bovine serum albumin) containing 2-3 ng of ^{35}S-RNA probe were applied on each section. Hybridization was carried out in humid chambers for 3.5 hours at 50°C. Post-hybridization procedures included washes in 50% formamide/2x SSC at 52°C, treatment with RNase A, and rinses in buffer. After an overnight wash in 2x SSC containing Triton-X-100, slides were rinsed, dehydrated, delipidated and air dried. Autoradiography was carried out using NTB-3 emulsion (Kodak). Exposure times were chosen according to results obtained in test slides developed at regular intervals. Slides were then developed (D-19, Kodak), fixed and counterstained with hematoxylin and eosin. Experimental and control tissues were processed according to the same conditions and at the same time, and developed together. Sections were examined using bright and dark-field microscopy.

Radioimmunoassay

L4 and L5 spinal cord segments and DRGs were removed from deeply anesthetized rats, 15 (n=5) and 60 (n=5) days after neonatal right sciatic nerve section, frozen on dry ice, divided into right and left sides, and stored at -70^{o}C. The tissues were thawed at 4 ^{0}C, homogenized in a solution containing protease inhibitors (1mM phenylmethyl-sulfonyl fluoride (PMSF), 1% aprotinin and 25 μM leu-peptin), and then processed for RIA according to a protocol suggested by Incstar, Inc. (Stillwater, MN) using a specific ^{125}I SP RIA kit.

SP-IR and CGRP-IR cell counts

The procedures have previously been described in detail (Himes and Tessler, '89). With the Bioquant Image Analysis system (BIAS), neuronal nuclei were counted in every twelfth section (12 μm-thickness) on (i) cresyl-violet-stained sections to determine the total number of neurons per DRG, and (ii) adjacent immunoreacted sections to determine the number of either SP-IR or CGRP-IR neurons. A factor for corrected neuron counts (Smolen et al., '83) was used to determine total and SP-IR and CGRP-IR neuron numbers. With the same BIAS system, the size of SP- and CGRP-IR neurons was determined by measuring the area of immunoreactive perikarya that contained a nucleus in the section plane. This measurement was carried out on all immunoreactive perikarya present in every 24th section.

Quantification of *in situ* hybridization labelling

The number of reduced silver grains associated with an individual cell was obtained using the Morphon Image Analysis System (Smolen and Beaston-Wimmer, '89) under bright-field illumination at high magnification (x40 lens). The area of interest was scanned and labelled cells identified. The perimeter of each labelled cell was traced, and both the area occupied by silver grains and the total area of the outlined cell were determined. The density of labelling was determined by area fraction (the area occupied by the grains divided by total area of the outlined cell) because the total area outlined

varied from one cell to other. The threshold level was adjusted to distinguish between grains and background (cell counterstain), and the measurement was carried out on all labelled cells with a nucleus present in the section plane.

RESULTS

The consequences of neonatal sciatic nerve lesion were similar in the L4 and L5 spinal segments and in the L4 and L5 DRGs. The results from both DRGs and both spinal cord segments were therefore pooled. The intact contralateral (left) spinal segments and DRGs were used as controls.

1. Selective survival of peptidergic neurons

A. Percentage of SP-and CGRP-IR neurons visualized in DRGs at birth:

The greater restoration of immunoreactivity in the dorsal horn after neonatal lesion could be accounted for if at birth a greater proportion of DRG neurons are peptidergic than in adults and if these are selectively spared.

Counts of peptidergic neurons in DRG from neonates indicate that ~25% of DRG neurons studied on the day of birth were immunoreactive for SP and ~50% were immunoreactive for CGRP (Fig.1). The percentage of these peptidergic DRG cells is thus the same on the day of birth as in the adult.

B. Percentage of SP- and CGRP-IR neurons surviving in DRGs after neonatal sciatic lesion :

Death of large numbers of neurons after neonatal sciatic nerve section-ligation greatly decreases the size of the DRG ipsilateral to the lesion (Fig. 2, 3). Cell counts of total DRG neurons show that 2 months after neonate axotomy, about 50% of cells die. Very similar data have been reported previously (Himes and Tessler, '89).

SP-IR neurons: The radioimmunoassay results show that SP levels recover to normal levels in the dorsal horn while the SP in the DRG is only about half that found in intact DRGs (Fig. 4). SP-IR is found in a subpopulation of small neurons that are scattered throughout both control and experimental DRG (Fig. 2). Quantitative cell size analysis (Fig. 5) demonstrates no significant difference in mean cell size between experimental and control DRGs (mean size=444 μm^2), and experimental and control DRG show the

same percentage of SP-IR neurons (25%; Fig. 1). Therefore, the 50% neuron death induced by neonatal axotomy does not selectively spare SP neurons.

CGRP-IR neurons: CGRP immunoreactivity in intact DRG is found mostly in small and medium size neurons, but also in some large cells (Fig. 3). The immunostaining is generally dark within the perikarya of small cells and lighter and more granular in the perikarya of larger cells. In the experimental DRG, 2 months after neonatal sciatic nerve lesion, CGRP immunoreactivity is also found primarily in small- and medium-size neurons. The histograms of cell size distribution show that CGRP neurons in both intact and operated DRG form a more heterogenous size population than SP-IR neurons, indicated by the the presence of more than one peak (Fig. 7). The mean cell size of CGRP neurons in the operated DRG is less than in control (518 μm^2 versus 678 μm^2). In both experimental and control DRGs, however, the percentage of CGRP-IR neurons is about 50% and there is no significant difference between the sides (Fig. 1). Therefore CGRP-IR neurons are not selectively spared by sciatic nerve section/ligation on the day of birth. In contrast, after sciatic nerve section in adult, only 20% of DRG neurons die (Himes and Tessler, '89), but the number of CGRP-IR neurons appears to be greatly decreased (Fig. 6).

2. Differences in peptide synthesis

β-PPT and α-CGRP mRNA in DRGs

DRG cells containing β-PPT or α-CGRP mRNA were identified with *in situ* hybridization histochemistry. In both operated and intact sides, specific autoradiographic labelling consists of clusters of reduced silver grains. Each cluster is associated with an individual cell, which can also be recognized by eosin-hematoxylin counterstaining. The neuronal populations labelled for β-PPT or α-CGRP are scattered throughout the DRG and correspond to SP- and CGRP-IR cells observed with immunocytochemistry. Neurons hybridized for α-CGRP-mRNA are a more heterogeneous population in size and more numerous than neurons hybridized for β-

PPT-mRNA (Fig. 8,9). To determine whether DRG neurons that survive neonatal sciatic nerve section increase their constitutive levels of peptide synthesis and in this way contribute to peptide recovery in dorsal horn, we quantified the number of reduced silver grains per neuron. In spite of their heterogenous size CGRP-IR neurons exhibit the same level of hybridized α-CGRP-mRNA because the same cell density of reduced silver grains is found. Two months after neonatal sciatic nerve section, the levels of hybridized β-PPT- as well as α-CGRP-mRNA in surviving DRG neurons do not differ from those found in the intact neurons of the contralateral DRG (Fig. 10). SP and CGRP gene expression therefore recover to normal levels, but do not exceed normal levels, in DRG neurons that survive neonatal sciatic lesion, even when regeneration is prevented. Recovery is therefore not attributable to a change in constitutive expression of these genes.

3. Apparent restoration is not an artefact attributable to shrinkage of the dorsal horn

Consequences of neonatal sciatic nerve lesion on SP- and CGRP-IR in the dorsal horn

SP immunohistochemistry and radioimmunoassay: In intact adult rats, SP-IR fibers in the dorsal horn are distributed homogenously in laminae I, II and the dorsal part of lamina III (Fig. 11). Sixty days after neonatal sciatic nerve section-ligation, the distribution of SP-IR fibers in the dorsal horn ipsilateral to lesion and the density of labelling are comparable to those of the contralateral intact dorsal horn (Fig. 11a; see also Himes and Tessler, '89). After neonatal lesion, the areas corresponding to laminae I and II ipsilateral to the lesion are smaller than on the control side. Quantitative results obtained by radioimmunoassay, however, show that both sides contain the same amount of SP (Fig. 4) and the restored staining is therefore not an artefact of shrinkage. In contrast, 2 months after sciatic nerve section-ligation in adults, the SP-IR is greatly reduced in the medial portion of laminae I and II, which receives input from the sciatic nerve (Fig. 11b).

CGRP immunohistochemistry: In the intact adult lumbar spinal cord CGRP-IR fibers are concentrated in laminae I and II and extend into lamina V, where they form a reticulated network (Fig. 12 b, c). No CGRP-IR perikarya are seen in the dorsal horn, although motorneurons are immunostained. Sciatic nerve section-ligation in the adult completely eliminates CGRP-IR in the medial portion of the dorsal horn (Fig. 12 c). This reduction is maintained for at least 2-4 months after lesion (latest survival time studied). In the neonate, CGRP-staining is reduced at 6 days after sciatic nerve transection (Fig. 12a), but by two months CGRP-IR fibers are present in the medial portion of laminae I and II with a pattern and level of staining that are virtually identical to those of the intact contralateral dorsal horn (Fig. 12 b).

4. Restoration occurs through central sprouting by injured or spared DRG neurons.

When the sciatic nerve is recut 2 months after neonatal lesion, a new depletion of both SP- and CGRP-IR occurs in the dorsal horn, particularly in the medial portion of laminae I and II (Fig. 11c, 13a). The decrease is less than that occurring after an adult lesion. This pattern suggests that L4 and L5 DRG neurons contribute substantially to, but are not the exclusive source, of recovered peptides. Both peptides are also depleted from the dorsal horn when L2, 3, 6 and S1 but not L4 and L5 dorsal roots are cut 2 months after neonatal lesion (Fig. 11d, 13b). In this case also the depletion is incomplete, although it is not confined to the medial portion of the dorsal horn. Therefore, sprouting by intact neurons in adjacent DRGs also contributes to SP- and CGRP-IR recovery. The new depletion caused by dorsal rhizotomy is smaller in the L5 spinal segment than in L4, even if the same number (n=2) of roots are cut above L4 and below L5. The restoration after neonatal lesions is therefore due to sprouting by adjacent spared roots and increased supply from the axotomized DRG neurons.

DISCUSSION

Our results support previous studies that showed that the consequences of sciatic nerve section/ligation in neonates differ from those occurring after the same injury in adults (Bondok and Sansone, '84; Yip et al., '84; Fitzgerald and Vrbova, '85; Himes and Tessler, '89; Fitzgerald et al., '90). We find that, despite the more extensive death of DRG neurons in newborns, SP-IR recovers rapidly and completely in the dorsal horn, whereas recovery in adults is slow and incomplete (Himes and Tessler,'89). The present results with RIA confirm the immunocytochemical data and provide additional evidence that this recovery is not an artefact due to shrinkage. The present data demonstrate that CGRP-IR also recovers in the dorsal horn after neonatal sciatic nerve section/ligation. The recovered SP- and CGRP-IR originate both from surviving axotomized neurons in the L4 and L5 DRGs and from intact neurons in DRGs rostral and caudal to L4 and 5. Similarities in recovery support the idea that the two neuropeptides are subject to common regulatory mechanisms.

1. Source of trophic support.

The mechanism responsible for the recovery of SP and CGRP synthesis in newborn DRG neurons permanently separated from their peripheral targets is unknown. DRG neurons can maintain baseline levels of neuropeptide synthesis without the addition of neurotrophic factors (Lindsay et al., '89), but recovery is likely to depend on an adequate supply of these factors. The neurotrophins NGF, neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and brain-derived neurotrophic factor (BDNF), are known to be essential for the survival of developing DRG neurons in vitro (Lindsay et al., '85; Maisonpierre et al., '90b; Hohn et al., '90; Berkemeier et al., '91; Hallbook et al., '91; Ip et al., '92), and this has also been shown for NGF in vivo (Johnson et al., '80; Hamburger et al., '81). NGF is produced by peripheral targets (Davies et al., '87), internalized at axonal endings, and transported retrogradely to the cell bodies of DRG neurons (Korsching and Thoenen, 1983; Richardson and Riopelle, '84; Yip and Johnson, '84), where it modulates

SP and CGRP expression (Lindsay and Harmar, '89; Lindsay et al., '89) and continues to provide trophic support for mature DRG neurons although no longer required for survival (Yip et al., '84). BDNF and NT-3 are also retrogradely transported by subsets of DRG neurons (DiStefano et al., '92), and mRNAs coding for neurotrophins are present in skin (Maisonpierre et al., '90a,b; Acheson et al., '91; Schecterson and Bothwell, '92), a target of SP- and CGRP-containing DRG neurons (O'Brien et al., '89). Anti-NGF antibodies administered to post-natal rats markedly reduce SP levels in DRG and dorsal horn (Otten et al., '80; Mayer et al., '82), and application of NGF to the proximal stump of transected nerve prevents SP-IR and CGRP-IR depletion (Kessler and Black, '81; Csillik, '84, Fitzgerald et al, '85; Inaishi et al., '92). The reduction in SP and CGRP levels that follows axotomy is therefore accounted for by interruption of retrogradely transported neurotrophins. Recovery of peptide levels in neonates when regeneration is prevented suggests the availability of adequate levels of neurotrophic factors from new sites. Axotomized nerves of newborns may also be more responsive to these factors than those of adults, e.g., more receptors in newborns (Buck et al., '87) or have more efficient retrograde transport (Raivich et al., '91). The factors may be supplied by Schwann cells or other non-neuronal cells in the peripheral nerve proximal to the injury, the neuroma, or the spinal cord. Whether peptide synthesis in DRG is subject to autocrine/paracrine regulation (Schecterson and Bothwell, '92) remains to be demonstrated.

Following sciatic nerve lesion, non-neuronal cells increase NGF mRNA synthesis proximal to the site of the injury (Heumann et al., '87; Lindholm et al, '87; Hengerer et al., '90; Matsuoka et al., '91). BDNF mRNA levels increase to a far greater extent than NGF mRNA levels distal to nerve transection, but do not increase proximally (Meyer et al., '92). Axotomized DRG neurons therefore remain exposed to NGF and perhaps other neurotrophins, although the amounts may be decreased. The more extensive DRG neuron death that follows sciatic nerve section in newborns would decrease the competition for these factors, thus ensuring that supplies of neurotrophic factors are

adequate to maintain peptide synthesis in those that survive. Therefore the supply of neurotrophins, which are insufficient to maintain peptide synthesis after adult lesion, may be adequate after neonatal lesion. The induction of NGF receptor synthesis by newly available neurotrophic factors may also contribute to recovery (Lindsay et al., '90; Miller, et al., '91).

In adult DRG neurons, the synthesis of CGRP differs from that of SP because it is regulated via retrograde transport from the the central as well as the peripheral process (Jessell et al., '79; Noguchi et al., '89; Henken et al., '90; Villar et al., '91; Inaishi et al., '92). Whether the expression of either SP or CGRP is regulated via the central process in newborns has not been determined, but higher levels of the mRNAs for NGF, BDNF, and NT-3 have been demonstrated in the newborn spinal cord than in the adult (Maisonpierre et al., '90a; Ernfors and Persson, '91).

2. Mechanisms of recovery.

L4 and L5 DRG neurons axotomized on the day of birth contribute substantially to the recovery of both SP and CGRP in the dorsal horn because recutting the sciatic nerve two months after the original lesion induces a new depletion. The present results make unlikely several other mechanisms that could account for the contribution made to recovery by axotomized DRG neurons.

a. Selective survival of SP- and CGRP-containing DRG neurons. Previous studies showed that peripheral axotomy, in adult and neonate, results in death of DRG neurons without a change in the soma size distribution, suggesting that both small and large cells are equally vulnerable (Cavanaugh, '51; Risling et al., '83; Heath et al., '86; Himes and Tessler, '89). In our present study, the cell counts based on immunocytochemical characterization demonstrate directly that there is no selective cell death in the subclasses of DRG neurons that are SP- or CGRP-IR. These results differ from those observed when developing DRG neurons are exposed either to capsaicin (Jancso et al., '77; Nagy et al., '81; Skofitsch and Jacobowitz, '85a; Carr et al., '90) or NGF-

antiserum (Goedert et al., '81; Kessler and Black, '81; Raivich and Kreutzberg, '87; Ruit et al. '92). Both of these treatments induce selective cell death within small size neuron populations. A shift in the size spectrum because of a decrease of mean cell size has also been observed after peripheral nerve axotomy (Aldskogius and Arvidsson, '78; Himes and Tessler, '89). Our results are consistent with these observations. We find no change in the distribution or the mean of SP cell size, whereas the cell size distribution of the more heterogeneous CGRP neurons lacks the peak corresponding to the largest cell population and shows a slight decrease in the mean cell size. This is likely due to slight atrophy of cell bodies or to loss of the largest cells which are few in number. We cannot rule out selective loss of some of the largest CGRP-containing cells, but selective survival of peptidergic neurons cannot account for recovery in the dorsal horn.

b. Change in neurotransmitter phenotype. Plasticity in neurotransmitter phenotype expression by DRG neurons is shown by the transient expression of catecholaminergic staining during development (Jonakait et al., '84) and by a sensitivity to target-derived influences observed in developing neurons in culture (Barakat-Walter et al., '91; Duc et al., '91) and in the regenerating nerves of adult rats in vivo (McMahon and Gibson, '87). Our data do not support the hypothesis that DRG neurons, which are incapable of SP or CGRP synthesis under normal conditions, change their neuropeptide phenotype in response to neonatal axotomy and produce these peptides. Because we found that the proportion of SP- and CGRP- neurons in the total neuronal population and their cell size distribution after neonatal lesion are comparable to those observed in the intact newborn and adult DRG, change in the phenotype is unlikely to account for recovery in the dorsal horn.

c. Increased peptide synthesis regulated at the level of transcription or translation. Decreases of SP and CGRP in the DRG and dorsal horn after peripheral nerve injury in the adult are related to decreased amounts of their mRNAs (Nielsch and Keen, '89; Noguchi et al., '89; '90; Henken et al., '90). If regeneration is unsuccessful,

levels of PPT-mRNA remain low due to decreased amounts of mRNA per neuron and a decrease in the number of neurons expressing PPT-mRNA that exceeds the percentage of loss of these cells (Henken et al., '90). The present results show that regulation of CGRP and SP synthesis after neonatal sciatic nerve lesion differs from that previously observed after adult lesion. Recovered DRG neurons exhibit normal levels of PPT- and CGRP- *in situ* hybridized mRNA per neuron and the percentages of these neurons immunoreactive for SP and CGRP are similar to those observed in intact adult DRG neurons. The total number of these neurons is, however, reduced by 50% compared with normal, and our results using RIA show a corresponding 50% reduction in levels of SP in the L4 and 5 DRG at a time when levels in the dorsal horn are completely recovered. Because constitutive levels of hybridized mRNA do not increase and levels of peptide synthesis correspond to the numbers of surviving DRG neurons, increased peptide synthesis cannot account for peptide recovery in the dorsal horn.

d. Other compensatory mechanisms. The present experiments have not directly addressed several additional mechanisms by which axotomized DRG neurons may contribute to the recovery of peptide levels in the dorsal horn. One of these mechanisms is the sprouting or other central compensatory events elicited in the central axons of these neurons after sciatic nerve injury which may be greater in neonates than in adults (Woolf et al., '90; Coggeshall et al., '91; Knyihar-Csillik et al., '92). Two metabolic adaptations in response to injury may also contribute: increased rates of peptide transport along the dorsal roots and decreased rates of turnover of the peptides in the central terminals. Changes consistent with a slowed rate of transport of the neurofilament/microtubule protein wave have been observed along the dorsal root after peripheral axotomy (Oblinger and Lasek, '88), but changes in the central transport of rapidly transported peptides like SP and CGRP and their metabolism within the spinal cord have not been examined (Keen et al., '82).

Recutting the sciatic nerve depletes the recovered peptides from the dorsal horn, but the depletion is incomplete. Additional sources must therefore contribute to the recovery. Because CGRP in the dorsal horn derives entirely from the dorsal roots (Gibson et al., '84; Chung et al., '88), the additional sources must include neurons in DRGs adjacent to L4 and L5 whose central processes undergo collateral sprouting in response to partial denervation of the shared targets in the dorsal horn. Our observation that cutting two dorsal roots rostral and two dorsal roots caudal to L4 and 5 depleted SP- and CGRP-IR that had recovered after newborn sciatic nerve section confirms this prediction. In adult animals sprouting by primary afferent axons, including those immunoreactive for CGRP (Piehl et al., '92), has been observed after dorsal rhizotomy (reviewed in Goldberger et al., in press), and myelinated primary afferents have been shown to extend into novel regions of the dorsal horn after peripheral nerve section/ligation (Woolf et al., '92). Sprouting in adults is in general more limited spatially than that which occurs in neonates (reviewed in Goldberger et al., in press). Several procedures, including the administration of NGF-antiserum to embyros (Ruit et al., '92) and capsaicin to newborns (Shortland et al., '90; Hammond and Ruda, '91), cause intact primary afferents to sprout into denervated regions of the spinal cord, and sprouting by saphenous dorsal root afferents into areas normally supplied by central projections of sciatic nerve afferents has been shown previously after neonatal sciatic nerve lesion (Fitzgerald, '85; Fitzgerald and Vrbova, '85; Shortland and Fitzgerald, '91). Unlike the recovery of SP- and CGRP-IR that we observed, however, the recovery of flouride resistant acid phosphatase (FRAP) staining in the dorsal horn that followed sciatic nerve section and ligation in newborns was incomplete and was not depleted by resectioning the sciatic nerve, indicating that FRAP recovery was entirely due to spared afferents (Fitzgerald and Vrbova, '85). SP is found in many of the same DRG neurons that contain CGRP (Gibson et al., '84; Skofitsch and Jacobowitz, '85b; Ju et al., '87), but the populations of SP- and FRAP-containing DRG neurons are largely separate (Nagy

and Hunt, '82), and those that contain FRAP and CGRP only partially overlap (Carr et al., '90). The differences in recovery between FRAP and SP and CGRP staining therefore suggest that the capacity for sprouting or metabolic compensation differs among subsets of neurons. In adult rats, collateral sprouting by the peripheral processes of high-threshold nociceptive axons has been shown to exceed that of low-threshold mechanoceptive axons (Jackson and Diamond, '84).

Conclusion

In spite of the extensive death of DRG neurons that follows sciatic nerve section/ligation in newborns, levels of SP- and CGRP-IR recover completely in the dorsal horn. Sprouting by neurons in DRGs adjacent to those whose axons are cut accounts for some but not all of the recovery. DRG neurons that survive axotomy also contribute, but not because of selective survival of SP- or CGRP-containing neurons, change in neuron phenotype or an increase in constitutive levels of peptide synthesis. These neurons appear to be supplied with sufficient quantities of neurotrophic factors to permit them to restore their normal levels of peptide production. The contribution of metabolic alterations in the neonatally injured neurons, including increased peptide transport or prolonged peptide half-life, remains to be determined.

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LEGENDS FOR FIGURES

Fig. 1. Bar graph representations showing the percentages of SP- and CGRP-IR neurons in the DRG of intact newborns (white bar) and adults (black bar), and in the DRG of adults 2 months after ipsilateral sciatic nerve section as a newborn (hatched bars). Neonatal and adult control DRG do not differ, nor do adult and operated DRG (paired comparison t-test) with respect to numbers of SP- or CGRP-IR neurons.

Fig. 2. Photomicrographs showing SP-IR in L5 DRG neurons of adults with right sciatic nerve section as a newborn. (a) Left (control) DRG. (b) Right (experimental) DRG. Note difference in size of ganglia and in number of stained cells on the control and experimental sides. x55.

Fig. 3. Photomicrographs showing CGRP-IR in L4 DRG neurons of adults 2 months following right sciatic nerve section as a newborn. (a) Left (control) DRG. (b) Right (experimental) DRG. x55.

Fig. 4. Bar graph representations of RIA results showing SP-IR in left (black bar) and right (hatched bar) L4 and L5 DRG and spinal cord segments 15 and 60 days after right sciatic nerve section as a newborn. Compared with the left side, levels of SP-IR are reduced in the right side DRG at both 15 (**, $p < 0.001$) and 60 days (*, $p < 0.05$) post-operative, but levels on the right side of the spinal cord do not differ (paired comparison t-test).

Fig. 5. Cell size histograms showing the distribution of SP-IR neurons in DRG of adults 2 months after right sciatic nerve section as a newborn. (A) Left (control) DRG. (B) Right (experimental) DRG.

Fig. 6. Photomicrographs showing CGRP-IR in L4 DRG neurons of an adult 2 months following right sciatic nerve section as an adult. (a) Left (control) DRG. (b) Right (experimental) DRG. x 55.

Fig. 7. Cell size histograms showing the distribution of CGRP-IR neurons in DRG of adults 2 months after right sciatic nerve section as newborns. (A) Left (control) DRG. (B) Right (experimental) DRG. The mean DRG neuron size is decreased on the experimental side.

Fig. 8. Dark-field *in situ* hybridization autoradiographs showing PPT mRNA in L5 DRG neurons 2 months after right sciatic nerve section as a newborn. (a) Left (control) DRG. (b) Right (experimental) DRG. x 80.

Fig. 9. Dark-field *in situ* hybridization autoradiographs showing CGRP mRNA in L4 DRG neurons 2 months after right sciatic nerve section as a newborn. (a) Left (control) DRG. (b) Right (experimental) DRG. x 80.

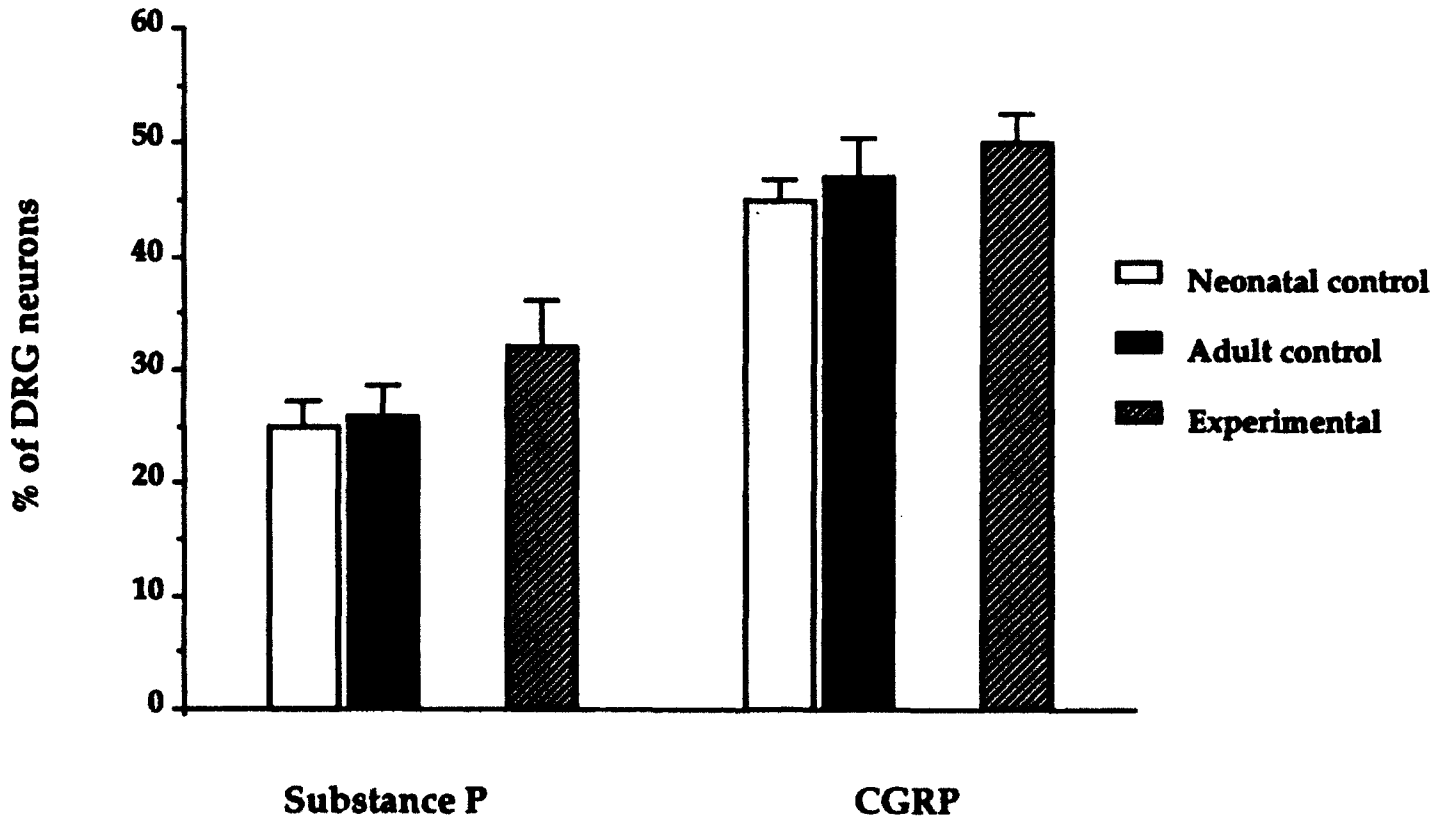
Fig. 10. Bar graph representation of *in situ* hybridization results showing the number of reduced silver grains over control (black bar) and experimental (hatched bar) L4 and L5 DRG neurons 2 months after right sciatic nerve section as a newborn. The number of grains is related to the amount of a-CGRP or b-PPT mRNA associated with an individual neuron. Area fractions are

calculated by: (area occupied by the grains per neuron)/ (total area of the neuron). Control and experimental sides do not differ (Student's t-test).

Fig. 11. Photomicrographs showing SP-IR in the left and right L4 or L5 dorsal horn. (a) 2 months after right sciatic nerve section as a newborn. (b) 2 months after right sciatic nerve section as an adult. (c) 2 months after right sciatic nerve section as a newborn followed by recutting the nerve and an additional 2 weeks survival. (d) 2 months after right sciatic nerve section as a newborn followed by right sided L2-L3 and L6-S1 dorsal rhizotomy and an additional 2 weeks survival. In (c) and (d) arrows mark areas of depletion on the experimental side. x 35.

Fig. 12. Photomicrographs showing CGRP-IR in the left and right L4 or L5 dorsal horns (a) 6 days or (b) 2 months after right sciatic nerve section as a newborn. (c) 2 months after right sciatic nerve section as an adult. Arrows in (a) and (c) indicate areas of depletion on the experimental side. x 35.

Fig. 13. Photomicrographs showing CGRP-IR in the right and left L4 or L5 dorsal horns 2 months after right sciatic nerve section as a newborn and 2 weeks after (a) Recutting the right sciatic nerve. (b) L2-L3 and L6-S1 right-sided dorsal rhizotomy. x 35.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50
40
30
20
10
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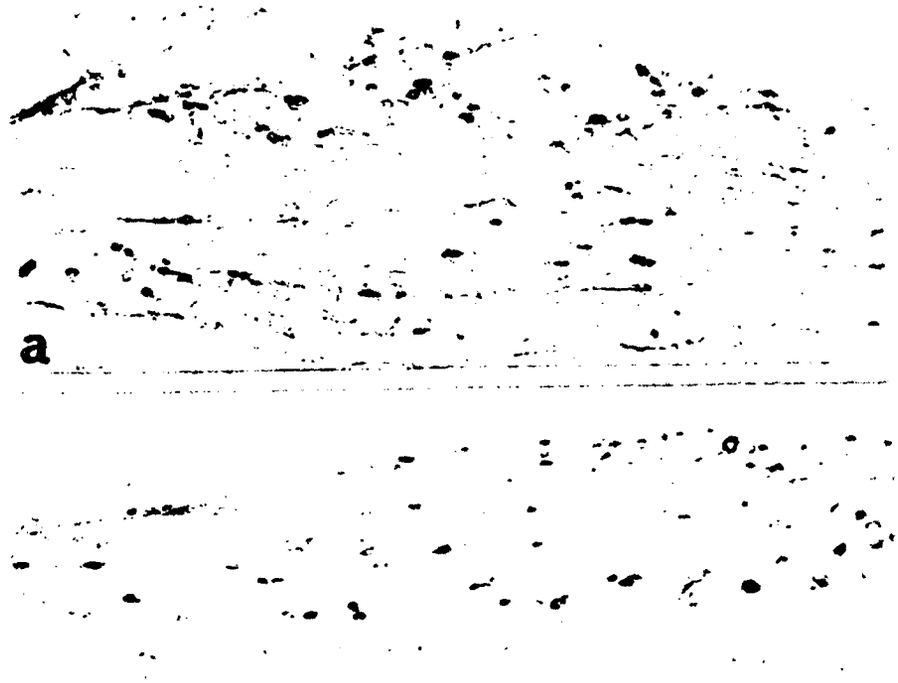
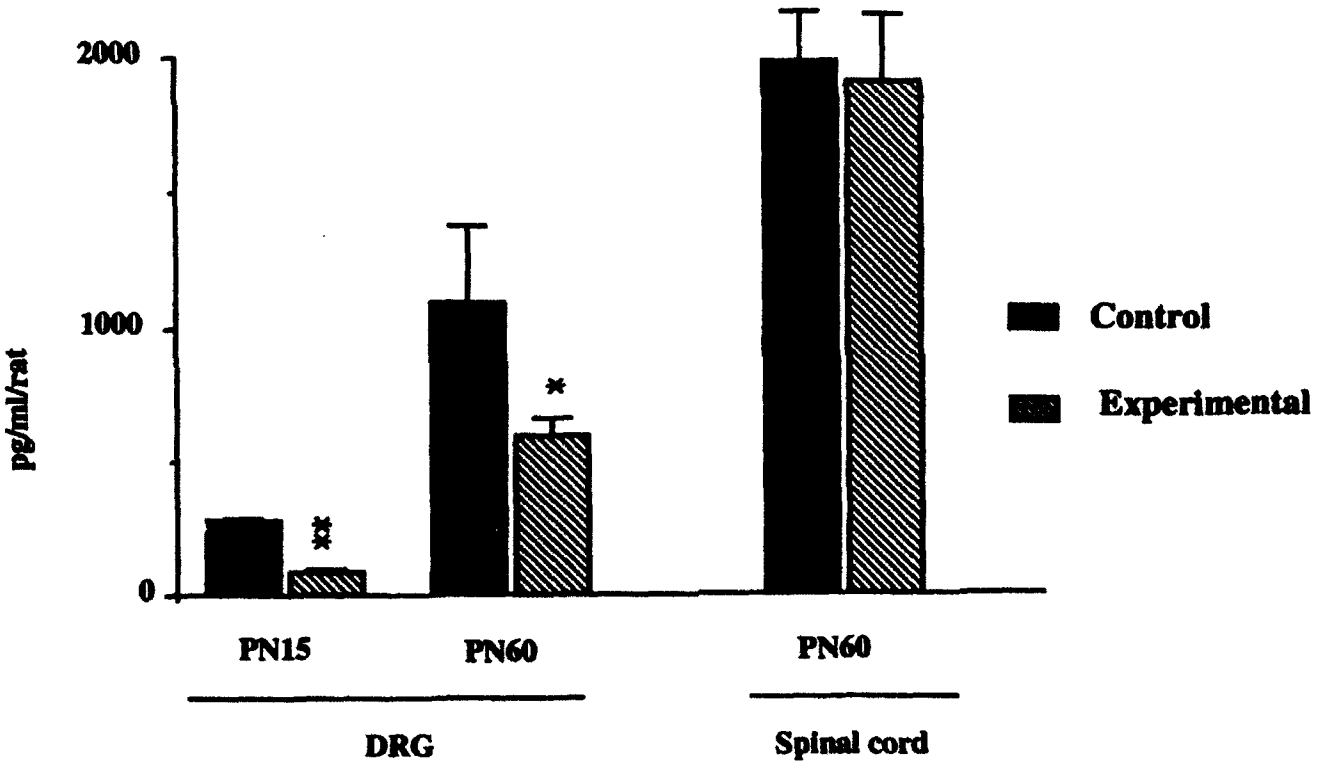
% of DRG neurons
Neonatal control
Adult control
Experimental
Substance P
CGRP


a
b

a

b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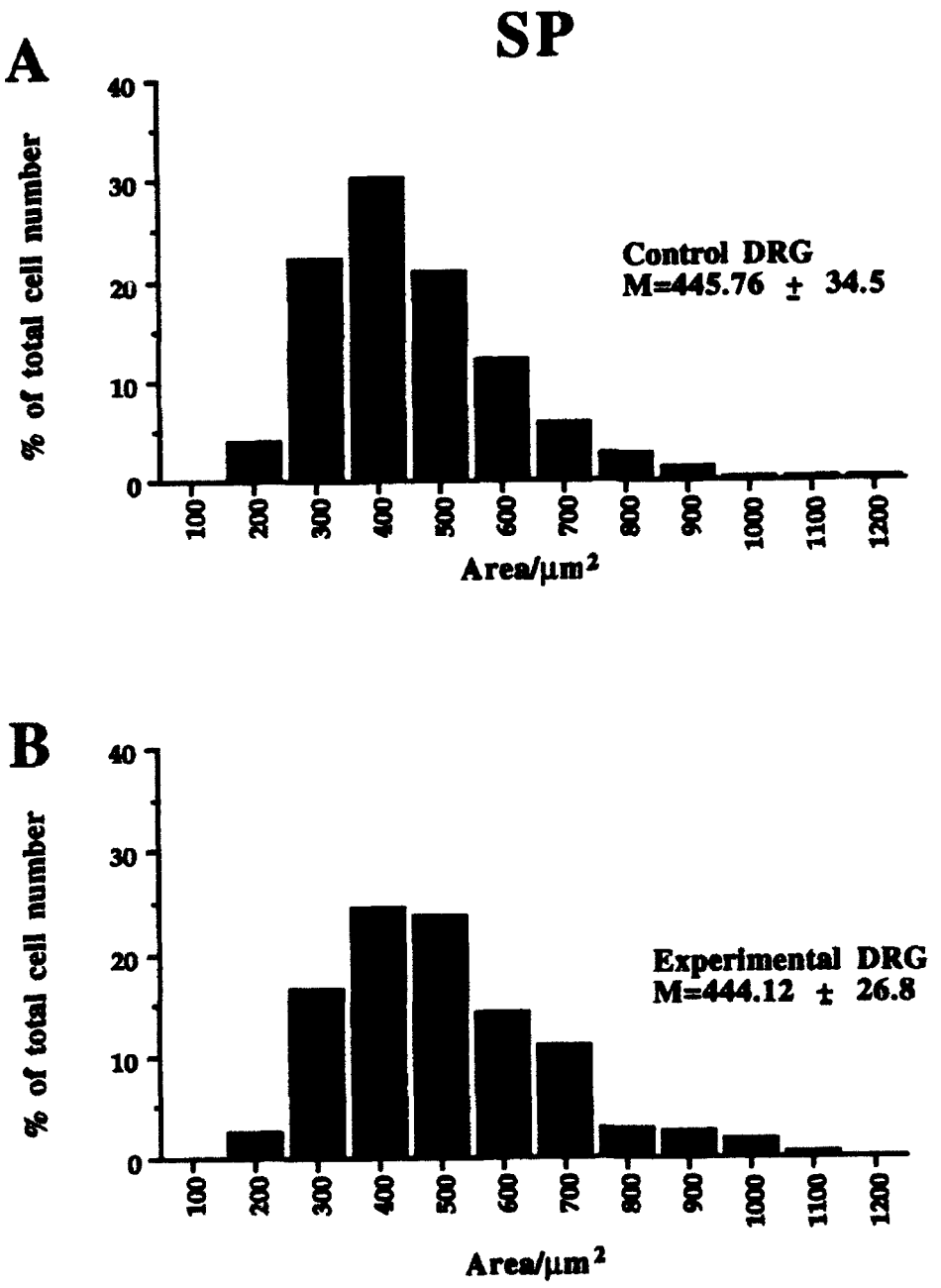
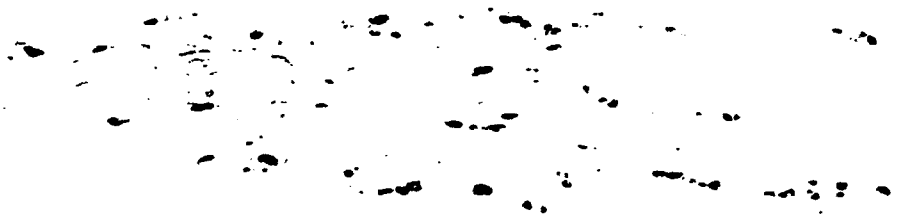
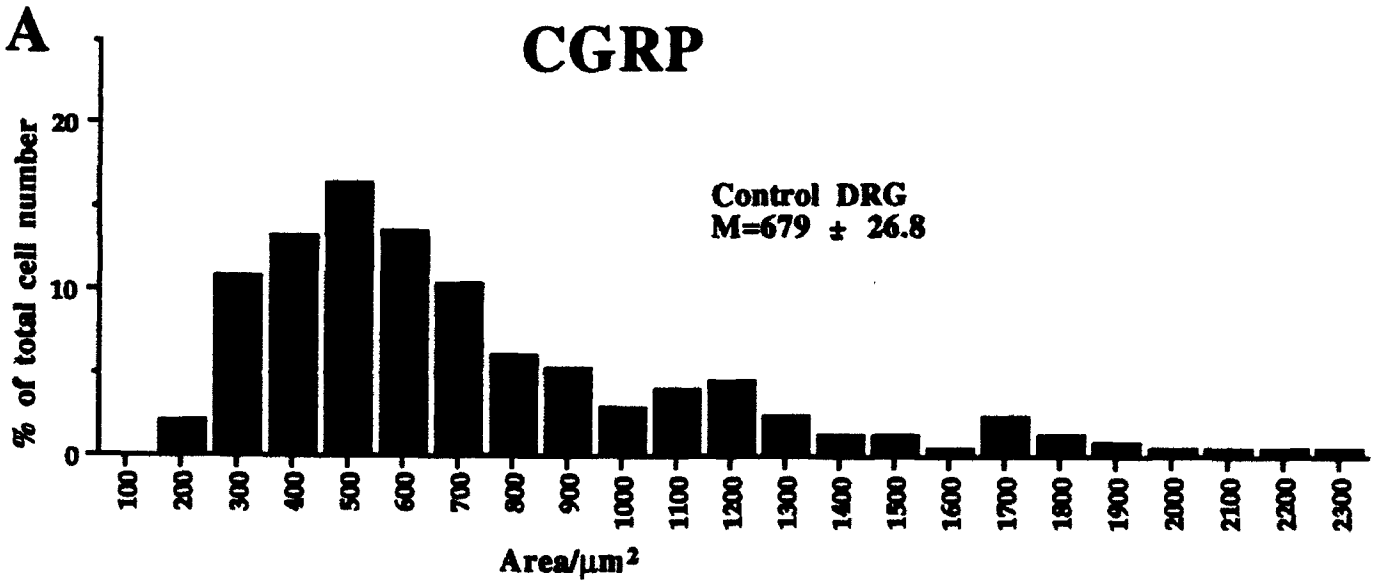
pg/ml/rat
**
*
Control
Experimental
PN15
PN60
PN60
DRG
Spinal cord


A
SP
% of total cell number
40
30
20
10
0
Control DRG
M=445.76 ± 34.5
100
200
300
400
500
600
700
800
900
1000
1100
1200
Area/μm²
B
% of total cell number
40
30
20
10
0
Experimental DRG
M=444.12 ± 26.8
100
200
300
400
500
600
700
800
900
1000
1100
1200
Area/μm²

a



b


A
CGRP
% of total cell number
20
10
0
Control DRG
M=679 ± 26.8
100
200
300
400
500
600
700
800
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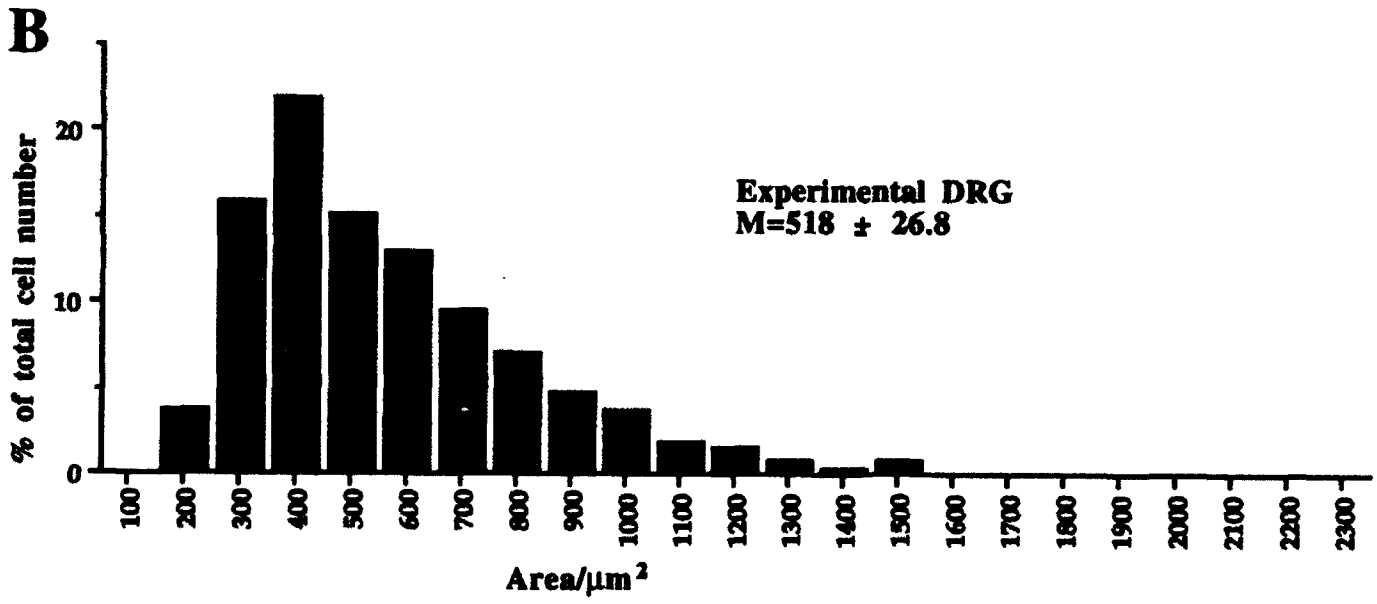
1000
1100
1200
1300
1400
1500
1600
1700
1800
1900
2000
2100
2200
2300
Area/μm²


B
% of total cell number
20
10
0
Experimental DRG
M=518 ± 26.8
100
200
300
400
500
600
700
800
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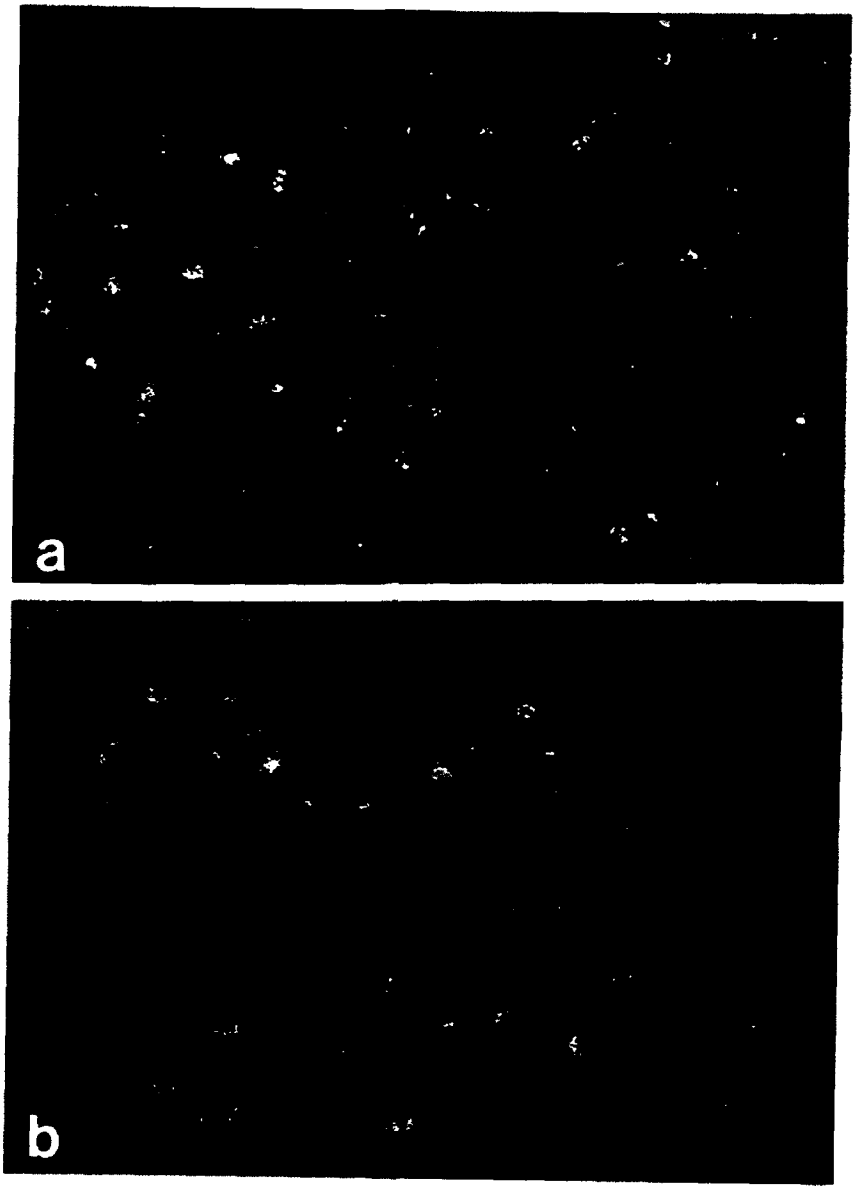
1700
1800
1900
2000
2100
2200
2300
Area/μm²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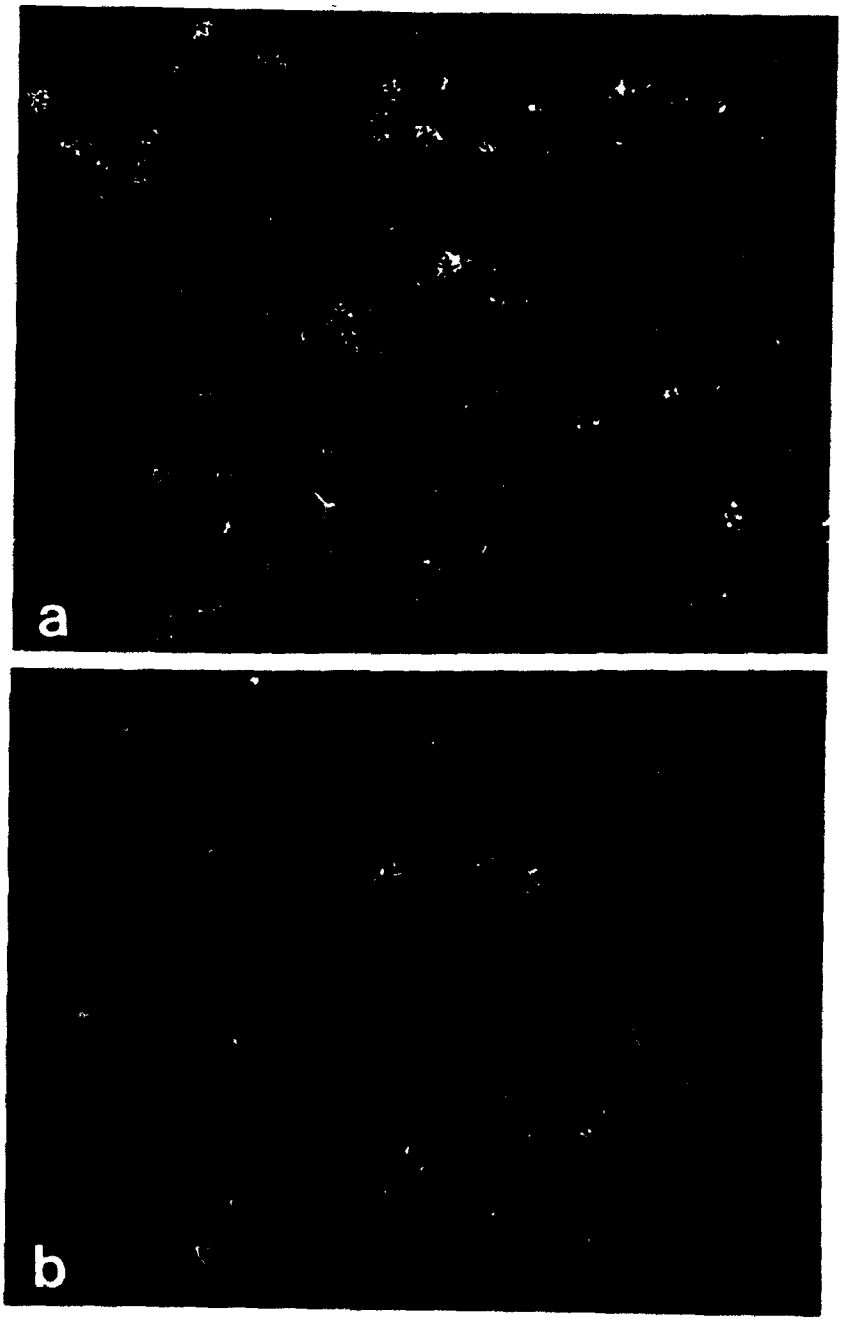
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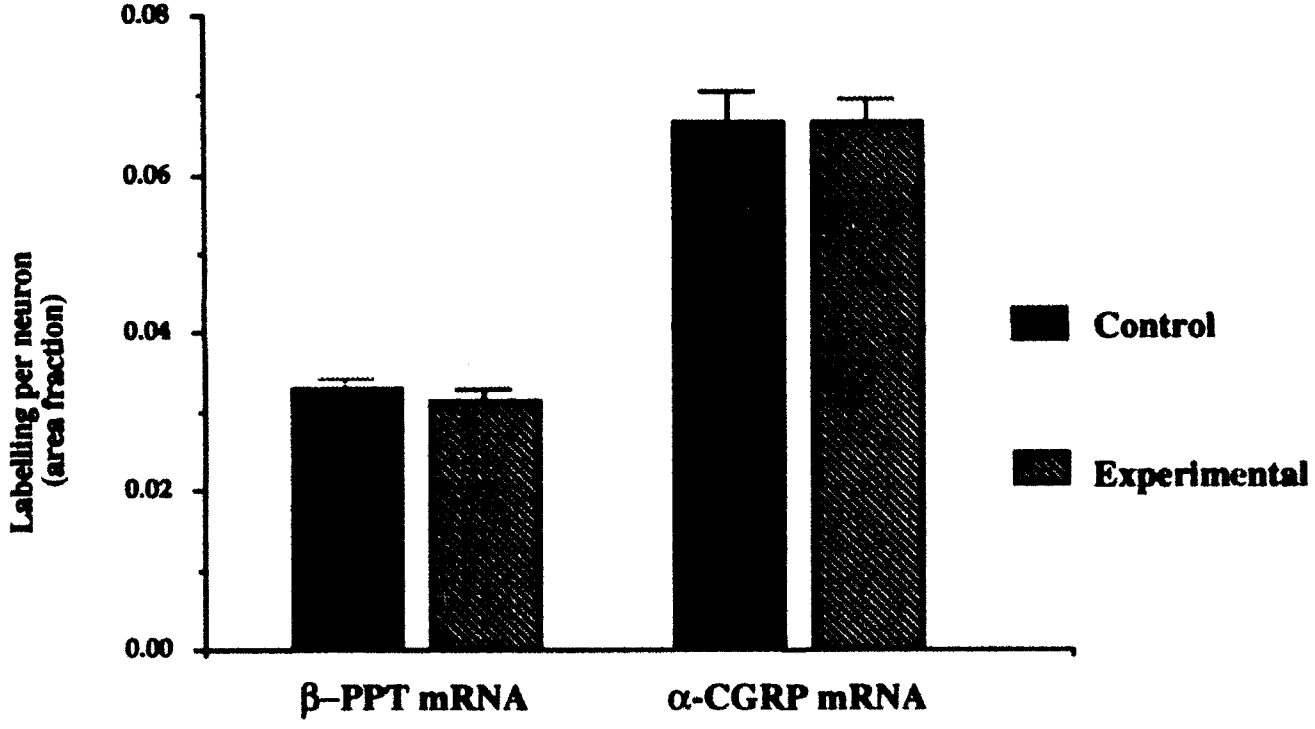
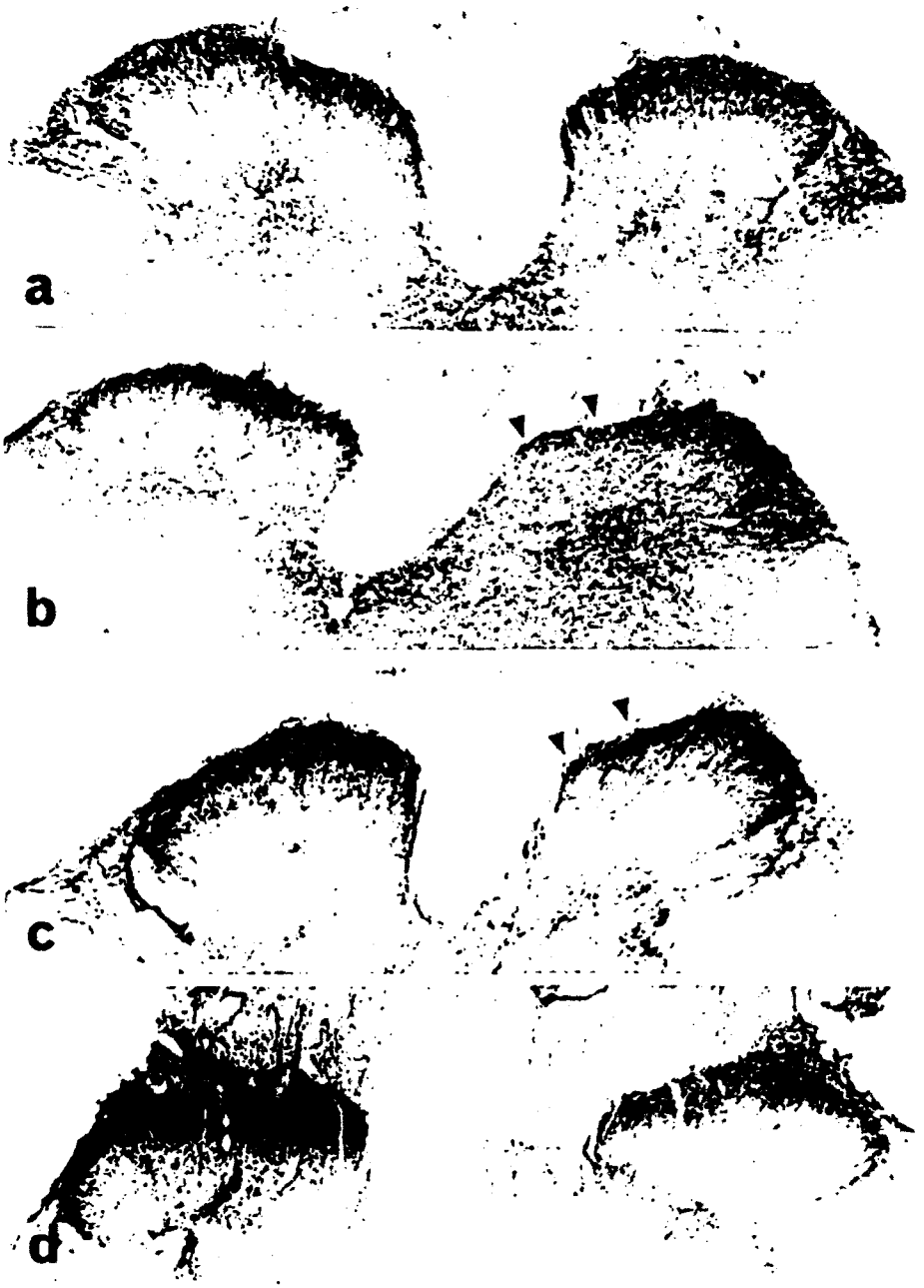
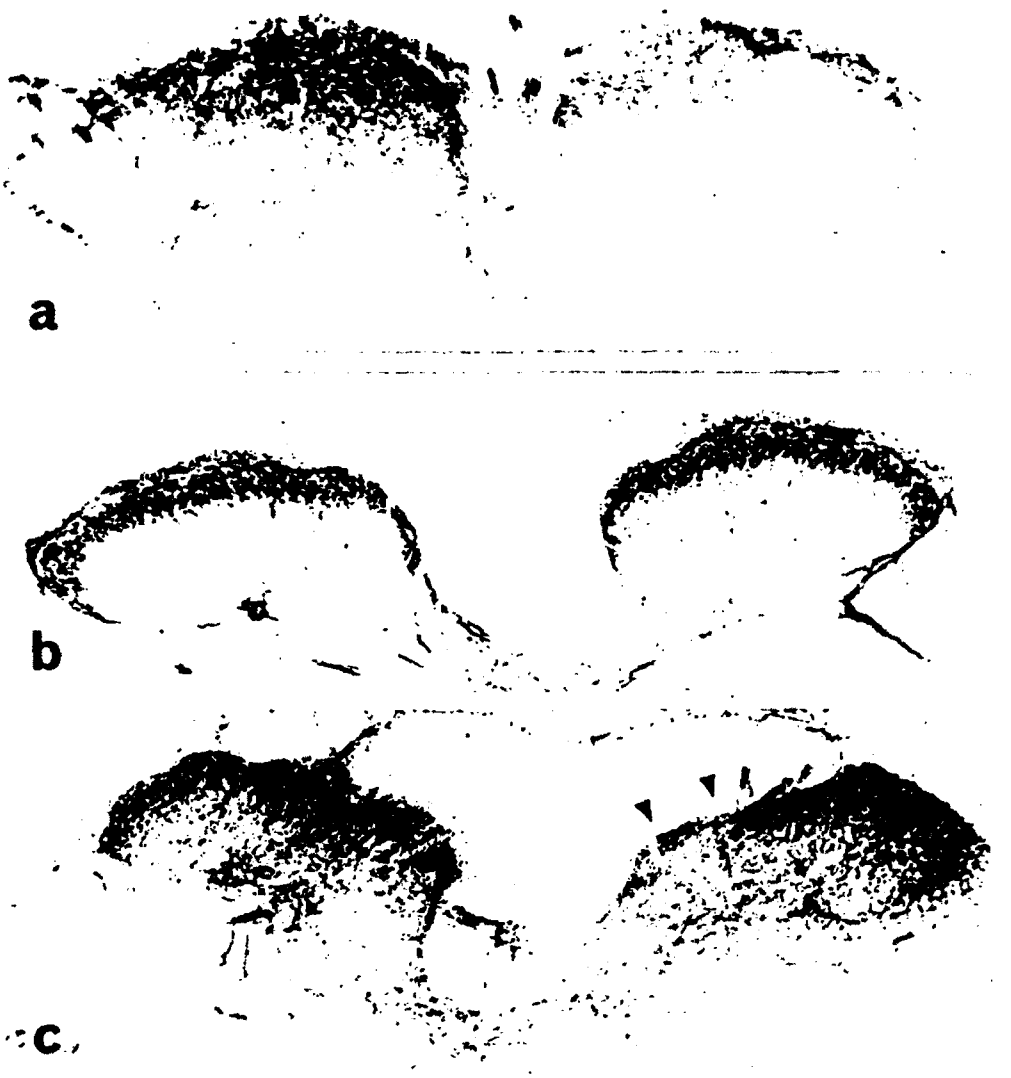

0.08
0.06
0.04
0.02
0.00
Labelling per neuron
(area fraction)
β–PPT mRNA
α-CGRP mRNA
Control
Experimental


a
b
c
d


a
b
c


a

b

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In situ hybridization of mRNA for β-preprotachykinin and preprosomatostatin in adult rat dorsal root ganglia: comparison with immunocytochemical localization

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Summary

In situ hybridization histochemistry was used to identify neurons in rat dorsal root ganglia that contained mRNAs encoding β-preprotachykinin and preprosomatostatin. The distribution of these neurons was compared with the distribution of neurons containing tachykinins or somatostatin, identified using immunocytochemical techniques. Neurons labelled for β-preprotachykinin mRNA constituted 20% of the total neuronal population and belonged to the small cell class. Neurons labelled for preprosomatostatin mRNA with either RNA or DNA hybridization probes constituted approximately 10% of the total cells and comprised a small cell group that differed in average size from the β-preprotachykinin labelled population. The distribution of cells containing tachykinin- or somatostatin-like immunoreactive material was identical to the distribution of cells containing the respective mRNAs and, in addition, individual somata in adjacent sections contained both the mRNA precursor and the peptide. These results suggest that for these neuropeptides the sensitivity of the two methods is equivalent and the respective mRNAs and peptides are co-localized in the same neurons.

Introduction

Somatostatin and the tachykinin, substance P, are two widely distributed peptides which are thought to function as neurotransmitters or neuromodulators in both the central and peripheral nervous system (reviewed in Hökfelt *et al.*, 1982; Johansson *et al.*, 1984). Multiple forms of somatostatin have been identified throughout the central nervous system (Epelbaum, 1986). Of the three molecular species that predominate, somatostatin-14 is the most abundant (Epelbaum, 1986), but smaller amounts of somatostatin-28 [an amino-terminally extended form of somatostatin-14 (Pradayrol *et al.*, 1980; Schally *et al.*, 1980)] and somatostatin-$28_{(1\text{-}12)}$ have also been demonstrated (Benoit *et al.*, 1984). All of these forms are synthesized by cleavage from a larger precursor molecule (prosomatostatin) which, together with a signal sequence (preprosomatostatin), is the translation product of a single gene (reviewed in Habener, 1981, 1987). Substance P is the most thoroughly studied tachykinin in neurons of the central nervous system (Ogawa *et al.*, 1985; Arai & Emson, 1986), but other tachykinins have also been identified, including substance K (neurokinin A, neuromedin L), neurokinin B (neuromedin K) and neuropeptide K (Kimura *et al.*, 1985; Arai & Emson, 1986). Three tachykinins are derived from a single gene after the RNA is differentially spliced to produce at least three preprotachykinin mRNAs (Nawa *et al.*, 1983, 1984); α-preprotachykinin contains the amino acid sequence for substance P alone; β-preprotachykinin, for substance P, substance K, and neuropeptide K; and γ-preprotachykinin, for substance P and substance K (Krause *et al.*, 1987). Neurokinin B is the product of a separate gene (Krause *et al.*, 1987).

Somatostatin and substance P are among several neuropeptides that are found in dorsal root ganglion cells (Hökfelt *et al.*, 1976; Price, 1985; Tuchscherer & Seybold, 1985). On the basis of their appearance in Nissl-stained material, dorsal root ganglion neurons have been subdivided into a small (400–600 μm^2) darkly-staining class and a large (> 800 μm^2) lightly-staining class (Lawson, 1979; Price, 1985). The small

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cell class accounts for between 75 and 80% of total dorsal root ganglion neurons (Lawson, 1979). Most peptidergic neurons among this class are thought to be involved in the processing of several kinds of sensory information, including nociception (Cervero & Iggo, 1980; Hunt & Rossi, 1985). Approximately 20% of the total population of dorsal root ganglion neurons have been shown to contain substance P-like immunoreactive product (27% of the small cell class), and 10% express somatostatin-like immunoreactivity (13% of the small cell class) (Hökfelt *et al.*, 1976; Price, 1985).

Little is known about the mechanisms by which the synthesis of peptides is regulated in dorsal root ganglion neurons, either under physiological conditions or in response to injury. This question could be approached directly if we could combine immunocytochemical methods that demonstrate the peptides with *in situ* hybridization histochemistry that labels specific mRNAs in individual neurons (Arentzen *et al.*, 1985; Lewis *et al.*, 1986; Goedert & Hunt, 1987; Chesselet *et al.*, 1987; Rethelyi *et al.*, 1987; Card *et al.*, 1988; Fitzpatrick-McElligott *et al.*, 1988; Baldino *et al.*, 1988a).

In the present study, we have used *in situ* hybridization histochemistry to study the distribution of mRNAs which encode β-preprotachykinin and preprosomatostatin in neurons of the dorsal root ganglion, and we have compared this distribution with the population of cells which contain tachykinin- or somatostatin-like immunoreactivity. Our results indicate that mRNAs for β-preprotachykinin and preprosomatostatin are expressed in two distinct populations of neurons. Our results further suggest that the neurons containing β-preprotachykinin mRNA are the same as those which synthesize tachykinins, and that cells which contain the preprosomatostatin mRNA are the same as those which synthesize somatostatin. Preliminary reports of these results have been presented elsewhere (Henken *et al.*, 1987a, 1987b).

Materials and methods

Tissue preparation

Adult female Sprague–Dawley rats (250–300 gm) were deeply anaesthetized with chloral hydrate (35–40 mg kg^{-1} intraperitoneally) and perfused intracardially with 50 ml normal saline, followed by 400 ml 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Dorsal root ganglia from the lumbar region (L4–L5) were dissected free, placed in 30% sucrose buffer overnight, then embedded in mounting medium (Tissue Tek, Miles) and kept frozen (−20° C) until sectioning. Sections (10 μm) were cut on a cryostat, thaw-mounted on triply subbed slides (porcine gelatin and chromalum) and thoroughly dried at room temperature. Adjacent sections were placed on two series of slides. One series was used for immunocytochemistry and maintained at 4° C until processed. The other series of adjacent sections was used for *in situ* hybridization histochemistry and was stored at −70° C.

Immunocytochemistry

Antisera to somatostatin and substance P were purchased from INCSTAR (formerly ImmunoNuclear, Inc., Stillwater, MN). The antiserum to somatostatin is directed against the 14-amino acid peptide somatostatin-14. Since somatostatin-14 is present at the C-terminus of both prosomatostatin and somatostatin-28, reaction product may indicate the presence of any of these forms. The antiserum to substance P appears to be directed against the N-terminal amino acids common to tachykinins, since the antiserum reacts strongly with eledoisin, physalaemin, and substance K, as well as with substance P. Reaction product found with this antiserum therefore reveals tachykinins, but is not specific for substance P.

Sections were labelled on slides either by the peroxidase–antiperoxidase (PAP) method of Sternberger (1986) or by the avidin–biotin method of Hsu *et al.* (1981a, 1981b). For the PAP method, 0.1% Triton-X-100 was added to all washes and serum dilutions, and sections were reacted with substance P antibody raised in rabbit at 1 : 1000 dilution. For the avidin–biotin method, mounted sections were reacted with rabbit anti-substance P or rabbit anti-somatostatin at 1 : 1000 dilution and then treated with biotinylated goat anti-rabbit IgG (Vectastain ABC Kit, Burlinham) and avidin–biotinylated horseradish peroxidase complex (Vectastain ABC) as specified by the manufacturer.

Controls for the specificities of both anti-substance P and anti-somatostatin immunocytochemistry consisted of substituting normal rabbit serum or blocked antiserum for substance P or somatostatin antiserum in the staining procedure. Blocked antiserum was produced by adding 100 μg of substance P or somatostatin to 1 ml of diluted substance P or somatostatin antiserum.

RNA probes: synthesis and in situ *hybridization*

Two cDNAs were used as templates: clone cHSPp11 is a 345-base pair cDNA for preprotachykinin containing exons 2 to 6 of the gene, isolated from a human brain library (Affolter *et al.*, 1985); the clone used for preprosomatostatin is a 400-base pair cDNA encoding preprosomatostatin sequences, isolated from rat medullary thyroid carcinoma (Goodman *et al.*, 1982). Specificity of hybridization for both probes has been previously characterized by Northern blot analysis (Goodman *et al.*, 1982; Affolter *et al.*, 1985).

Both cDNAs were inserted into the transcription vectors pSP65 (Promega Biotech, Madison, WI), restricted appropriately, and transcribed according to the manufacturer's specifications using 2.5 μM ^{35}S-UTP (1000 Ci $mmol^{-1}$, New England Nuclear) and 10 μM unlabelled UTP. All other nucleotides were unlabelled and present in large excess. For preprotachykinin, cDNAs were inserted in either direction with regard to the SP6 promoter, resulting in the synthesis of both sense [identical to the cellular mRNA (control)] and antisense [complementary to the cellular mRNA (test)] RNA probes. Only the antisense mRNA was synthesized for preprosomatostatin.

Hybridization was performed according to the method of Chesselet *et al.* (1987). Briefly, mounted tissue was rinsed in

buffer, acetylated, treated with glycine in Tris HCl, dehydrated and air dried. Two to 5 ng/slide of ^{35}S-RNA probe in 25 μl of hybridization mixture [40% formamide, 10% dextran sulfate, 4 × SSC (1 × SSC: 0.15 M sodium chloride and 0.015 M sodium citrate), 10 mM dithiothreitol, 1 ng ml^{-1} sheared salmon sperm DNA, 1 mg ml^{-1} *Escherichia coli* tRNA, 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrolidone, 10 ml^{-1} bovine serum albumin)] were placed on each slide. Hybridization was carried out in humid chambers for 3.5 h at 50° C. Post-hybridization procedures included washes in 50% formamide/2 × SSC at 52° C, treatment with RNase A, and rinses in buffer. After an overnight wash in 2 × SSC containing Triton-X-100, slides were rinsed, dehydrated, delipidated and air dried. Autoradiography was carried out using NTB-3 emulsion (Kodak). Slides were placed in light-tight boxes and exposed at 4° C for 3–8 weeks, developed (D-19; Kodak), fixed and counterstained with haematoxylin and eosin.

DNA probe: synthesis and in situ *hybridization*

A 39-base oligonucleotide probe was synthesized according to previously published procedures (Arentzen *et al.*, 1985). This probe was complementary to the 3'-coding region of rat somatostatin mRNA and was 3'-end-labelled with ^{35}S-dATP and separated as described elsewhere (Lewis *et al.*, 1986). The probe has previously been shown to be sensitive to RNase treatment, to recognize a single species of RNA by Northern blot analysis, and to possess a T_m within 4° C of the theoretical T_m (Card *et al.*, 1988).

In situ hybridization was performed according to previously published procedures (Wolfson *et al.*, 1985; Davis *et al.*, 1986; Lewis *et al.*, 1986; Baldino *et al.*, 1988a, 1988b). Pre-hybridization steps included incubation of the mounted tissue in proteinase K (1 μg ml^{-1} in 20 mM Tris-HCl, 2 mM $CaCl_2$), delipidation, rehydration and rinses in distilled water. The tissue was incubated overnight at 37° C in 50 μl hybridization buffer (50% formamide, 10% dextran sulfate, 10 mM dithiothreitol, 4 × SSC, 0.1% SDS, 0.1% denatured salmon sperm DNA, 0.1% denatured yeast RNA, 10 × Denhardt's solution) which contained the ^{35}S-labelled probe (1.5×10^6 cpm). The following day the sections were washed in 2 × SSC and rinsed in decreasing concentrations of SSC containing 14 mM β-mercaptoethanol and 1% sodium thiosulfate to a final stringency of 0.5 × SSC, air dried and coated with NTB-2 emulsion (Kodak). Tissue was exposed at 4° C for 2–4 weeks in light-tight boxes, developed (D-19; Kodak), fixed and counterstained with cresyl violet.

Data collection and analysis

For analysis of results obtained with both *in situ* hybridization histochemistry and immunocytochemistry, longitudinal sections of dorsal root ganglia were outlined using a camera lucida. Every neuronal soma containing a nucleus in a section of dorsal root ganglion was traced. The circumferences of all neuronal perikarya were measured using a planimeter, the cross-sectional area was calculated, and the cell was scored as labelled or unlabelled. For immunocytochemical reactions, labelled cells were readily differentiated from unlabelled cells by the presence of a moderate to very dense brown immunoreaction product in the cytoplasm. For *in situ* hybridization histochemistry, a cell was scored as labelled if at least 10 reduced silver grains were present in the emulsion overlying the cytoplasm (background was approximately 1 grain/cell body). Three representative sections of a dorsal root ganglion were analysed for each of three animals. Thus, immunoreactive or hybridization positive cells were mapped and counted in nine sections of dorsal root ganglia for each of the following experiments: tachykinin immunocytochemistry, β-preprotachykinin mRNA *in situ* hybridization, somatostatin immunocytochemistry, and preprosomatostatin mRNA *in situ* hybridization.

Histograms representing the cross-sectional area of every cell were compiled in order to compare the distribution of the labelled cells with the total cell populations. The mean cross-sectional areas of the labelled cells were compared with the mean cell areas of the total dorsal root ganglion population for each of the four experiments. Results obtained with *in situ* histochemistry were compared to those obtained by immunocytochemistry and the ratios of the labelled-cell population and the total cell population were determined. Differences between mean proportions were analysed with Student's *t*-test.

Results

TACHYKININ

Preprotachykinin in situ *hybridization histochemistry*

In situ hybridization using the cHSPp11 labelled transcript identifies dorsal root ganglion cells which contain β-preprotachykinin mRNA (Fig. 1A). Cells hybridizing with the antisense probe are located throughout the dorsal root ganglia; no labelled cells are seen using the sense probe. The distribution of neurons expressing β-preprotachykinin mRNA does not show a dorsal-ventral or lateral-medial bias, although this was not systematically quantified.

The distribution of the population of neurons hybridizing with the probe for β-preprotachykinin mRNA as compared to the entire neuronal population is presented in the histogram in Fig. 2A. Neurons containing mRNA for β-preprotachykinin comprise an average of 17% of the total number of dorsal root ganglion somata in each dorsal root ganglion, and the distribution of these neurons is centred in the smaller range of cell areas. The labelled-cell areas range from 92 to 940 μm^2, with an average cross-sectional area of 420 μm^2, unlabelled cells range from 86 to 4378 μm^2 (see Table 1).

Tachykinin immunocytochemistry

Tachykinin-immunoreactive cells are distributed throughout the ganglion. Figure 2B illustrates the distribution of the labelled-cell population as a function of cell area, compared to the distribution of the total cell population. The labelled cells belong to the small cell class, with an average cross-sectional area of 460 μm^2; the range for labelled cells 120–1112 μm^2

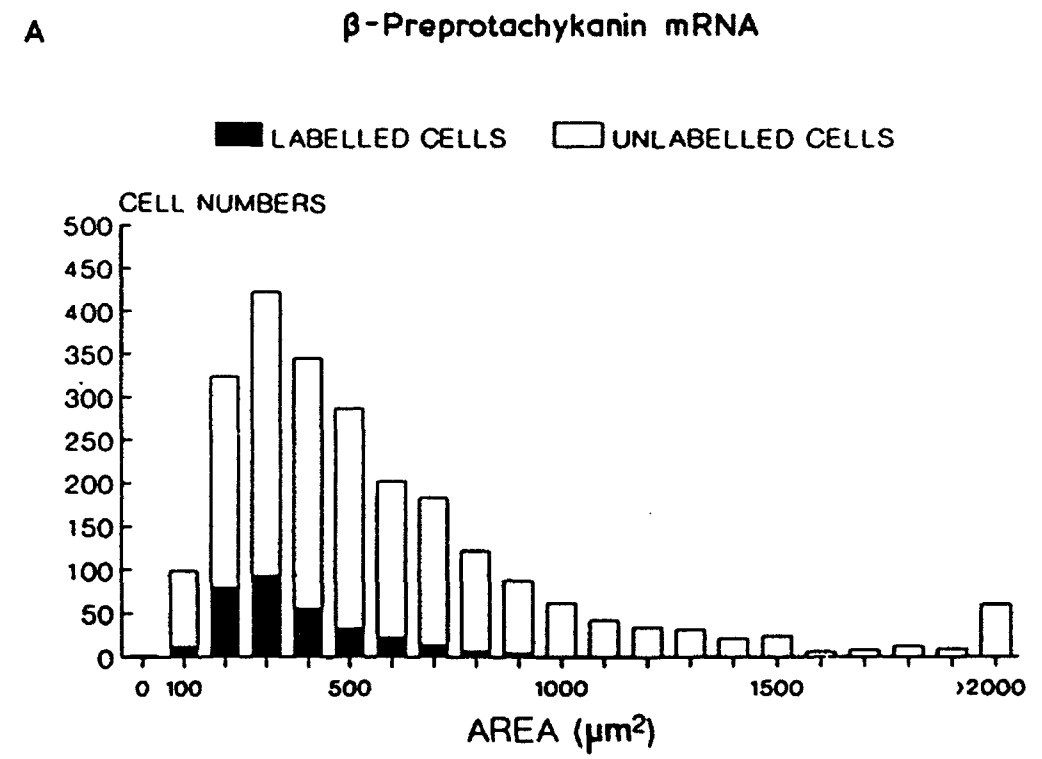
Fig. 1. (A) An *in situ* autoradiograph showing cells labelled for β-preprotachykinin mRNA (arrows). Silver grains are black and are localized in the emulsion overlying the somata. (B) An adjacent section stained immunocytochemically showing the same cells expressing tachykinin-like immunoreactivity (arrows). Magnification bar equals 25 μm.

Table 1. Summary of results (mean ± SEM)

	% Labelled cells/total cells	*Mean cross-sectional area of labelled cells (μm^2)*	*Size range of labelled cells (μm^2)*
TACHYKININ			
In situ histochemistry	17.4 ± 4.2	420 ± 12.9	92–940
Immunocytochemistry	21.0 ± 6.1	460 ± 9.6	120–1112
SOMATOSTATIN			
In situ histochemistry	10.0 ± 2.1	600 ± 16.9	240–1340
Immunocytochemistry	8.6 ± 2.0	653 ± 19.3	170–1840

whereas the total population of neurons ranges from 95 to 3980 μm^2. Approximately 21% of the neurons in each dorsal root ganglion are labelled (see Table 1).

A comparison of the ratios of hybridized or immunoreactive cells to total cell population shows that the percentages of cells labelled by the two methods are not different ($P > 0.05$). Moreover, comparison of the mean somal areas of cells labelled with *in situ* hybridization histochemistry with the areas of the immunocytochemically labelled neurons demonstrates that there is no statistical difference between the two populations ($P > 0.05$). Therefore, the two



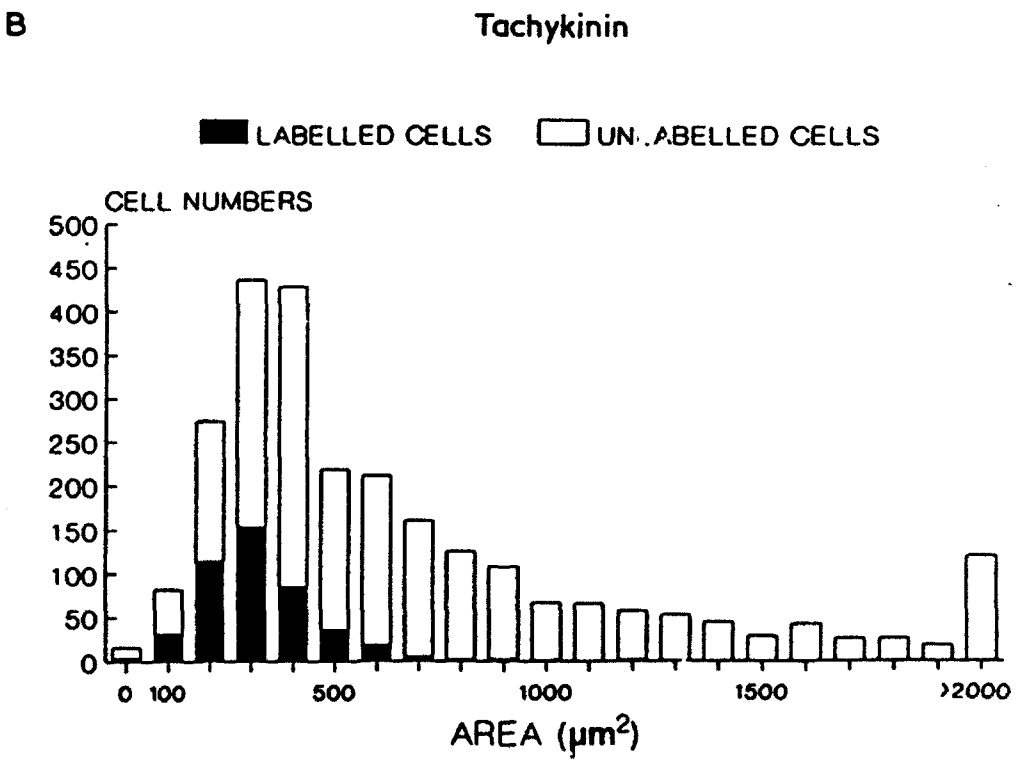


Fig. 2. Histograms showing the distributions of neurons labelled for β-preprotachykinin mRNA using *in situ* hybridization histochemistry (A) (cell number = 2050) and the distribution of neurons that contain the tachykinin-like immunoreactive product determined with immunocytochemistry (B) (cell number = 2247) in lumbar dorsal root ganglia. Black bars refer to labelled cells, white bars to the unlabelled population. The histograms illustrate that the populations labelled by the two methods have similar distributions.

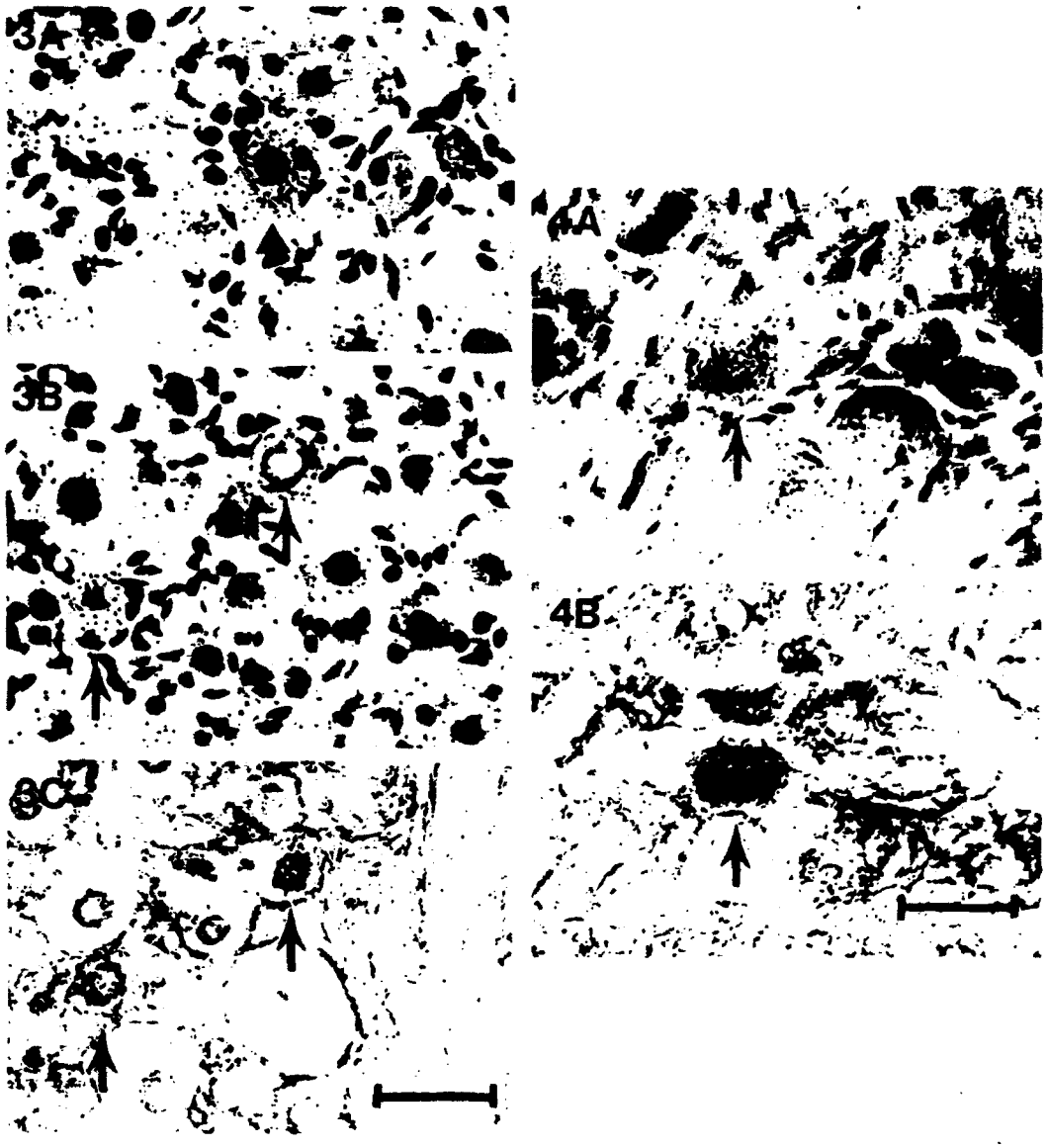


Fig. 3. (A) *In situ* autoradiograph of a densely labelled cell using an RNA probe for preprosomatostatin (arrowhead). Photomicrographs of adjacent sections of dorsal root ganglia showing two neuronal somata (arrows) labelled for the mRNA for the precursor peptide, preprosomatostatin (B) and the peptide itself (C). The *in situ* autoradiograph in (B) was obtained by hybridization with an RNA probe for preprosomatostatin. Magnification bar equals 25 μm.

Fig. 4. An *in situ* autoradiograph showing a cell labelled for the mRNA for preprosomatostatin using a DNA synthetic oligonucleotide probe (A). The same cell is shown stained with anti-somatostatin antibody (B) in an adjacent section. Magnification bar equals 25 μm.

techniques appear to be equally sensitive and to label the same cell populations.

In order to test this result, we used adjacent sections to determine whether single cells that contain mRNA for β-preprotachykinin are the same cells that contain tachykinin. Figure 1 shows neuron cell bodies seen in adjacent sections of a dorsal root ganglion that are labelled both autoradiographically for β-preprotachykinin mRNA (Fig. 1A) and immunocytochemically for tachykinin-like immunoreactivity (Fig. 1B). Thus, individual positive cells contain both the mRNA encoding the precursor peptide and the peptide itself.

SOMATOSTATIN

Preprosomatostatin in situ *hybridization histochemistry*

RNA and DNA probes identify cells containing mRNA for preprosomatostatin in dorsal root ganglia (Figs 3A, B; 4A). The RNA probe labels 9.8% of neurons with an average cell size of 615 μm^2, whereas the DNA probe labels 10.1% of the total cell population with an average cell size of 598 μm^2 (Fig. 5A). Statistical

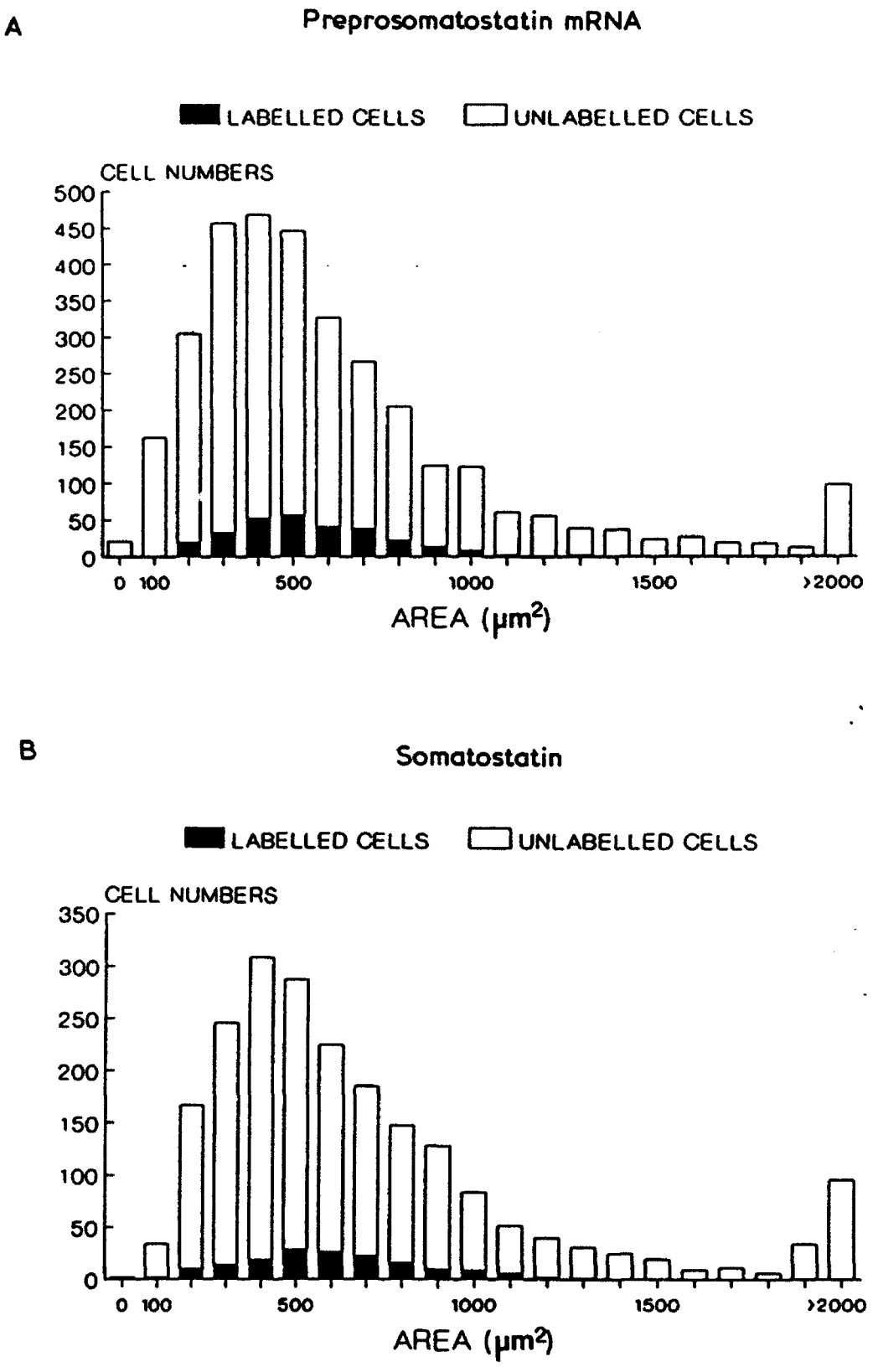


Fig. 5. Histograms showing the distributions of neurons labelled for preprosomatostatin mRNA using *in situ* hybridization histochemistry (A) (cell number = 2996) and the distribution of neurons labelled immunocytochemically with anti-somatostatin antibody (B) (cell number = 1968) in dorsal root ganglia.

analysis confirms equal sensitivity of the two probes ($P > 0.05$). Thus, data from the two were combined for all following analyses.

Approximately 10% of the total population of dorsal root ganglion neurons contain mRNA for preprosomatostatin. The average cross-sectional area of these cells is 600 μm^2, placing them in the small category of dorsal root ganglion neuronal somata. The areas of the labelled cells range from 240 to 1340 μm^2, while the total population of dorsal root ganglion neurons ranges from 104 to 4506 μm^2. Therefore, some somatostatin cells are larger than those labelled with the β-preprotachykinin probe, but they still belong to the small subclass of dorsal root ganglion neurons (see Table 1).

Somatostatin immunocytochemistry

The distribution of somatostatin-immunoreactive neurons is displayed in the histogram in Fig. 5B. Approximately 8.6% of the total population are labelled immunocytochemically. The histogram appears qualitatively similar to that seen in Fig. 5A for preprosomatostatin mRNA; labelled cells range in area from 170 to 1840 μm^2, while the total cells range in area from 98 to 4004 μm^2. The average area is 653 μm^2 (see Table 1).

Statistical comparison of the mean somal areas labelled with *in situ* hybridization histochemistry for preprosomatostatin mRNA as compared to immunocytochemistry for somatostatin does not show a significant difference ($P > 0.05$), nor does a comparison of the percentage of labelled cells ($P > 0.05$) across the two techniques, indicating that the same population of cells is labelled by the two techniques.

We examined adjacent sections of dorsal root ganglia labelled for preprosomatostatin mRNA and somatostatin immunocytochemistry in order to confirm that individual cells labelled by probes for preprosomatostatin mRNA also contain the somatostatin peptide. Labelling with RNA (Figs 3A, B) and DNA (Fig. 4A) probes was compared. Figure 3B shows an autoradiograph depicting two cells labelled with mRNA for preprosomatostatin hybridized with an RNA probe, while Fig. 3C shows the same two neurons stained for the peptide in an adjacent section. In Fig. 4A a cell labelled with a synthetic oligonucleotide DNA probe for preprosomatostatin is illustrated. In an adjacent section (Fig. 4B) the same cell is labelled for the peptide. Individual somata that contain mRNA for preprosomatostatin therefore also contain the peptide and this can be shown with equivalent sensitivity by both RNA and DNA hybridization probes.

Discussion

The principal findings of the present study are that mRNAs encoding neurotransmitter peptides can be visualized within individual dorsal root ganglion neurons by *in situ* hybridization histochemistry, and that neurons that produce the preprosomatostatin mRNA are the same as those which contain somatostatin-immunoreactivity; cells which produce one of the mRNAs for preprotachykinin contain tachykinin-immunoreactivity. The cell populations labelled by the two methods are similar, indicating equal sensitivities of the two techniques for these substances.

The discrete distribution of neuronal labelling observed, as well as the correspondence between the cells labelled with *in situ* hybridization histochemistry and those labelled by immunocytochemistry, strongly argues for the specificity of the hybridization in our experimental conditions. This is further confirmed by the absence of labelling in sections hybridized with a sense RNA probe identical to β-preprotachykinin mRNA, the difference in labelling obtained with RNA probes complementary to two different mRNAs (preprosomatostatin and β-preprotachykinin), and the observation that DNA and RNA probes for preprosomatostatin mRNA labelled the same population of neurons. The specificity of the labelling patterns is in agreement with previous observations that these probes label discrete populations of brain neurons corresponding to cells expressing immunoreactivity to somatostatin (Arentzen *et al.*, 1985; Chesselet & Robbins, 1987; Baldino *et al.*, 1988b; Card *et al.*, 1988; Fitzpatrick-McElligott *et al.*, 1988) or tachykinins (Chesselet & Affolter, 1987; Chesselet *et al.*, 1987). The direct demonstration of similar results with ^{35}S-radiolabelled RNA and oligonucleotide (DNA) probes indicates that, under the conditions employed, both probes display similar sensitivity for the detection of preprosomatostatin mRNA in dorsal root ganglion neurons. *In situ* hybridization, unlike Northern blot analysis, allows preprotachykinin and preprosomatostatin mRNAs to be detected and counted in single neurons while preserving the structure of the dorsal root ganglion. Northern blot analysis requires pooling of several dorsal root ganglia to obtain precise quantitation, with consequent loss of information about individual dorsal root ganglia or neurons (Tessler & Hudson, unpublished observations).

Our immunocytochemical results are in accord with previously published literature. Neurons immunoreactive for substance P comprise approximately 20% of dorsal root ganglion neurons (Hökfelt *et al.*, 1976; Price, 1985; Tuchscherer & Seybold, 1985) and those immunoreactive for somatostatin represent approximately 10% (Hökfelt *et al.*, 1976; Price, 1985). The mean cell size of somatostatin-immunoreactive dorsal root ganglion neurons is larger than that of neurons immunoreactive for substance P (see also Price, 1985), but both populations are within the size range of the 'small dark' dorsal root ganglion neurons which have been defined histologically (Hökfelt *et al.*, 1976; Price,

1985). The finding that the mean cell size of the neurons immunoreactive for somatostatin differs from that of neurons immunoreactive for tachykinin is consistent with previous suggestions that the two populations of neurons are largely distinct (Hökfelt *et al.*, 1976; Price, 1985), but does not exclude the possibility that somatostatin and substance P are co-localized in individual neurons, as has been reported for lumbar dorsal root ganglia of the cat (Johnson *et al.*, 1987).

The correspondence between these numbers and those obtained for the neuronal populations expressing β-preprotachykinin and preprosomatostatin mRNA strongly suggests that the respective mRNAs and peptides coexist in the same cell. This suggestion was confirmed by our demonstration of mRNA and peptide coexistence in cells studied in adjacent sections as has been shown by others (Uhl & Sasek, 1986; Goedert & Hunt, 1987). Therefore, the results indicate that expression of the specific genes encoding somatostatin and tachykinin precursors occurs in those neurons that contain the immunoreactive peptide. This rules out the possibility that a major portion of these peptides reaches the dorsal root ganglion neurons through an uptake process or were synthesized from mRNA species other than those recognized by the probes used in this study. Similar results for somatostatin in the central nervous system have also been reported (Fitzpatrick-McElligott *et al.*, 1988). However, in the case of tachykinins, neither the antibodies nor the hybridization probes can distinguish among the closely related peptide products or mRNAs produced from the preprotachykinin gene. At least three tachykinins, substance P, substance K and neuropeptide K, have been demonstrated in the rat central nervous system (Arai & Emson, 1986; Valentino *et al.*, 1986), and all of them are known to derive from an alternately processed primary transcript from a single gene (Krause *et al.*, 1987).

For the probes used in the present study *in situ* hybridization and immunocytochemistry label the same cells with comparable sensitivity. Studies from this and other laboratories have indicated that changes in the content of peptides in neurons following lesions can be demonstrated immunocytochemically. Substance P-containing primary afferents, in particular, show considerable plasticity in response to axotomy of their peripheral processes (Tessler *et al.*, 1985). It is not known whether the decrease in concentrations of substance P (Jessell *et al.*, 1979; Barbut *et al.*, 1981; Ogawa *et al.*, 1985; Tessler *et al.*, 1985) and substance K (Ogawa *et al.*, 1985) observed after axotomy is effected at the level of gene transcription, post-translational processing or both. The present results suggest that this question can be directly addressed by combining *in situ* hybridization histochemistry and immunocytochemistry to study the mechanisms that regulate metabolic changes during regeneration in individual neurons of the dorsal root ganglia.

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Appendix 18

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Grafts and functional recuperation

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Abstract

Plasticity of undamaged projections (axonal sprouting) in the adult and neonatal mammalian spinal cord has been documented many times. It has been associated, in some paradigms, with recovery of specific functions and motor behavior. This mutually occurring recovery of function appears to be enhanced by transplants of fetal tissue.

Introduction

CNS injury is followed by a period of severe depression of function which is then succeeded by some recovery of function. Spinal transection in adults interrupts the descending motor pathways [57], and results in a complete and permanent loss of descending control of motor function. There is also a transient depression of reflex function caudal to the lesion [14]. Eventually, some recovery of reflex locomotion, as elicited by a treadmill, can be demonstrated in the hindlimbs caudal to the transection [18,19]. The changes within the spinal cord caudal to the transection are likely to contribute to the reflex recovery [21]. Thus a permanent loss of voluntary movement and a transient loss of reflex function are seen after the same lesion. The recovery of reflex behavior implies plasticity in spinal segments below the lesion which have been indirectly affected by the transection. New studies using fetal transplants into injured spinal cord offer the possibility of enhancing this naturally occurring plasticity and achieving greater recovery of function.

Functional recovery

Naturally occurring plasticity in the spinal cord has been studied most thoroughly in 3 incomplete spinal lesion preparations: hemisection, spared root, and deafferentation in adult cats [23]. In each, the pathways that mediate recovery of motor behavior are the same pathways that show anatomical plasticity (sprouting).

Spinal cord hemisection

The first phase of recovery after spinal hemisection in the cat consists of return of basic locomotion on a platform and of crudely performed, high threshold postural reflexes such as placing. The second phase consists of refinement of the postural reflexes and a decrease in the thresholds for eliciting the reflexes. The change in threshold may be due to a compensatory increase in afferent control of movements when descending control has been interrupted. Collateral sprouting of dorsal root afferents occurs after hemisection [22,44] and the sprouting of dorsal root terminals may represent a mechanism underlying the increase in reflex control.

There are also physiological changes after hemisection that are consistent with a compensatory increase in afferent control after a loss of descending input. The strength of the monosynaptic stretch reflex and the cutaneous flexor reflex is increased [31,32] and there is an increase in the number of spinal neurons responding to cutaneous input [11,12,52] after hemisection.

Spared root preparation

The pattern of recovery after spared root deafferentation is similar to that after hemisection: a lowering of the threshold for postural reflexes is associated with recovery of accurate limb placement and the return of a normal kinematic pattern used in locomotion. In both preparations, dorsal root sprouting may act to increase the afferent control of movement and in this way ac-

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count for the recovery of motor behavior. Spared descending pathways could contribute to recovery in both cases, but most of the recovered behavior remained after additional hemisection, suggesting that primary afferent control was largely responsible for recovery after both types of injury. This suggests that there may be a competitive interaction among the spared pathways after a lesion and that they do not contribute equally to the recovery process.

The physiological changes after partial spinal cord deafferentation are consistent with the idea of increased afferent control mediated by the spared root in response to partial denervation of its terminal field. There is a loss of responsiveness in the lateral dorsal horn acutely after an L6 spared root deafferentation followed by a recovery of responsiveness, but the receptive field organization has changed in this portion of the dorsal horn [53]. Proximal receptive fields were permanently lost, and this loss was compensated by an increase of mixed receptive fields. This suggested the strengthening of weak afferent input which might be mediated by sprouting or other mechanisms. Mendell et al. [42] recorded from spinocervical tract cells after L7 spared root deafferentation and also found a loss of responsiveness when peripheral areas supplied by the spared root were stimulated. There was a subsequent recovery of responsiveness due to an increase in high-threshold mechanoreceptive input. Thus although there was recovery, the recovered input was not the same, or in the same proportions, as the original normal input.

Hindlimb deafferentation

To examine the contribution of dorsal roots to loss and recovery of motor function, the dorsal roots L_1-S_2 were cut on one side of the spinal cord [20]. The initial deficit is more severe than after hemisection or spared root lesions. The deafferented limb is not used in locomotion but is dragged behind as the animal walks on three legs. The descending reflexes, including the scratch and vestibular placing reflexes, are eliminated. All segmental reflex behavior, including crossed reflexes from the contralateral hindlimb, are abolished. Recovery begins soon, on the second post-operative day, and consists of participation of the deafferented limb in the step cycle for overground locomotion. The frequency of stepping is reduced from normal. The kinematic pattern is clearly abnormal and the steps of the deafferented limb are sometimes hypermetric and sometimes hypometric. During the first postoperative week there is some recovery of the descending scratch and vestibular reflexes. By the second post-operative week the limb can be used for accurate placement on a narrow (5 cm) runway. Although weight-bearing is somewhat deficient, the animals can place the limbs under the center of gravity and bear weight. Initially there are many errors but the number of errors decreases and the speed of crossing increases.

After hemisection or spared root deafferentation, the relationship between postural reflex recovery and recovery of locomotion could be examined. Since all afferent input has been eliminated on one side in the completely deafferented model, a similar analysis cannot be carried out. However, it is possible to compare descending control of locomotor recovery and reflex control. Overground locomotion depends on the presence of descending input. Quadrupedal treadmill locomotion requires descending input of the propriospinal system but does not require supraspinal control. Bipedal hindlimb locomotion requires only segmental systems and it recovers after complete spinal transection. When these three types of locomotion are examined after deafferentation, it becomes clear that the recovery is selective. Overground locomotion on the runway begins to recover during the first week. Quadrupedal locomotion on the treadmill begins to recover during the second week and the frequency of stepping of the deafferented limb is permanently reduced. Bipedal locomotion on the treadmill does not recover at all. This implies that activation of the deafferented limb requires descending supraspinal or propriospinal input but that segmental input activated by the contralateral hindlimb is inadequate. Thus recovery of locomotion is mediated by descending systems but not by segmental systems, or segmental systems by themselves cannot mediate locomotion after deafferentation.

This result was surprising considering that the spinal pattern generator for locomotion remained intact. We considered that after deafferentation, the deafferented limb might be dominated by some descending inhibitory system. If this were true, then removal of descending input after recovery from deafferentation might release the deafferented limb from inhibition and permit the return of bipedal hindlimb stepping. Therefore the spinal cord was transected after the recovery from deafferentation had reached a plateau. There was no subsequent recovery of locomotion in the deafferented limb and even the recovery of locomotion on the other side was slower and less extensive than in otherwise intact spinal cats. An alternative explanation for the results is that there is simply inadequate facilitation, after deafferentation, to activate locomotor circuits on the deafferented side when descending pathways are inactivated by the behavioral conditions (i.e. when bipedal rather than quadrupedal locomotion is tested) or by transection.

These behavioral observations cannot completely define the mechanisms underlying recovery. The anatomical patterns seen after chronic deafferentation are, however, consistent with the pattern of recovery. First, there is a complete recovery of terminal number in major target areas of the dorsal roots [43]. Second, when the distribution of staining using a monoclonal antibody specific for dorsal roots is examined, it is clear that the deafferented side remains depleted of dorsal root input and that dorsal roots from the intact side do not invade deafferented regions [22]. This is consistent with the observation that recovery does not depend on contralateral systems. Third, an interneuronal system containing substance P and a descending system containing serotonin both exhibit increases in transmitter content in the dorsal horn after deafferentation [60]. This is consistent with the idea that descending control is important in mediating recovery of locomotion after deafferentation.

Intraspinal transplants

The amount of recovery that occurs spontaneously is qualitatively obvious and quantifiable but nevertheless limited. This naturally occurring plasticity of spinal neurons can however be extended or enhanced by introducing transplants into damaged spinal cord.

Heterotypic transplants

Transplants of specific cell types have been inserted into a damaged spinal cord with the goal of restoring function mediated by those cell types. Monoamines administered systemically after spinal cord transection can activate the intrinsic spinal cord circuitry that mediates locomotor [17] or autonomic [41) function. Brainstem monoaminergic neurons also can restore function without reconstituting damaged neuronal circuits [4,46]. Both brainstem catecholaminergic neurons important for locomotion and serotonergic neurons important for autonomic function have been transplanted into the caudal region of transected spinal cord [48]. The transplanted noradrenergic locus coeruleus and serotonergic mesencephalic or medullary raphe neurons extend axons for up to 1–2 cm into host spinal cord [5,15,16,45,46], restore levels of neurotransmitter depleted by the transection [50,51], and terminate in the same regions as in normal spinal cord [45,50,51]. Transplanted serotonergic axons establish synapses on host motoneurons and neurons in the host intermediolateral column that are similar to those formed by brainstem serotonergic axons in normal spinal cord [50,51]. The projections of transplanted locus coeruleus neurons have not yet been examined.

Both types of transplanted brainstem monoaminergic neurons have been shown to contribute to recovery in experimental models of spinal cord injury. Transplants of embryonic raphe serotonergic neurons mediate recovery of reflex ejaculation in spinal rats [50,51]. Transplanted noradrenergic locus coeruleus neurons are thought to account for the recovery of hindlimb flexion reflexes in rats whose catecholamines have been chemically depleted [13] and for the recovery of reflex stepping activity in rats whose spinal cord has been transected [61]. These embryonic transplants therefore contribute to behavioral recovery although they have been placed in the spinal cord caudal to transection and cannot be regulated normally by the host or restore the damaged neuronal circuits. The activation of intrinsic spinal cord networks by the release of transmitter onto or in the vicinity of the normal targets of these neurons appears to be adequate to account for the recovery of these behaviors.

The recovery of other types of behavior lost after spinal cord injury is likely to require more faithful reconstruction of the damaged neuronal circuits. Additional strategies using peripheral nerve grafts or embryonic spinal cord transplants have therefore been developed. Such transplants may contribute to the restoration of function in at least 3 ways: (i) by rescuing axotomized neurons that would otherwise die; (ii) by serving as a conduit for the regrowth of damaged host axons directly across an area of damage; (iii) by serving as a site in which relays are established between neurons in host spinal cord and neurons in the transplant which may project to host neurons.

Segments of peripheral nerve rescue axotomized retinal ganglion cells [59] and when inserted into the spinal cord support the elongation of intraspinal axons [28,55,56]. The axons of dorsal root ganglion (DRG) neurons and those that originate from neurons whose perikarya are located close to the site of insertion appear favored to grow. The axons of supraspinal neurons that have been injured further from their perikarya are less likely to project into the grafts [56]. CNS axons can grow within the peripheral nerve graft for distances that exceed their normal length, and retinal axons establish synapses on normal target neurons that retain the normal morphological features and activate the target neurons (reviewed in [2]). After leaving the peripheral nerve graft, however, the CNS axons show very limited growth within the host parenchyma and terminate within 1–2 mm of the end of the graft [1]. Peripheral nerve grafts have contributed a great deal to our understanding of the importance of the neuron's environment for regeneration, but their contribution to

functional recovery after spinal cord injury has received little attention.

Homotypic transplants

80–90% of embryonic spinal cord transplants now survive in the acutely injured spinal cord of adult and newborn rats [3,54] and in the chronically injured spinal cord of adult rats [27]. Transplants also survive in the completely severed spinal cord [29,49]. Although they lack the characteristic butterfly shape of normal spinal cord gray matter, several morphological features of these transplants encourage the expectation that transplants can contribute to the reconstruction of interrupted neuronal circuits and replace damaged populations of spinal cord neurons. For example, areas develop within transplants that resemble substantia gelatinosa [35], supporting the hypothesis that transplants might function as relays. The astrocytic reaction that develops between transplant and host is interrupted by regions in which the tissues are apposed and processes pass from one to the other [54]. Transplants may also reduce the extent of the astrocytic scarring that follows spinal cord injury [27,38], which is thought to represent an obstacle to regeneration.

The connectivity between transplants and adult hosts has been studied by tract tracing and immunocytochemical techniques, and electrophysiological methods are beginning to be employed. Fetal spinal cord neurons transplanted into adult spinal cord form an extensive network of connections with one another, but few donor neurons extend processes into host spinal cord, and most of these terminate near the interface between transplant and host [36]. The number of host spinal neurons that extend processes into transplants is also limited, and the perikarya of most of these are located within 0.5 mm of the interface [36]. Only a few corticospinal [8,37] or serotonergic brainstem [54] axons are found within transplants placed into aspiration cavities made in adult spinal cord, and these axons also penetrate only a short distance into the transplants. In adult hosts, therefore, it seems unlikely that transplants can function as conduits that will allow regenerating axons to traverse a region of injury. When transplants are placed into excitotoxic rather than aspiration lesions where cells are killed but axons spared and the observed growth represents axonal sprouting rather than regeneration, central monoaminergic and dorsal root axons immunoreactive for calcitonin gene-related peptide (CGRP) grow more robustly than corticospinal or rubrospinal axons [47].

The possibility that embryonic spinal cord transplants might function as the site of relays between sets of injured adult axons has been tested by studying the ability of cut dorsal roots to regenerate into transplants [58]. The cut central processes of DRG neurons cannot regenerate into adult spinal cord in the absence of a transplant, but, when provided with a transplant, at least the subset of dorsal roots that contains CGRP grows in sufficient numbers to allow features of their growth to be analyzed quantitatively [26,33,34,58]. The terminals of regenerated CGRP-containing dorsal roots form synapses with transplant neurons; as in lamina I of normal dorsal horn, most of these are axodendritic and simple and complex synaptic contacts are present in proportions similar to normal [33]. Differences between the synapses formed in transplants and normal lamina I are also found. For example, regenerated CGRP-containing axons are significantly more likely to form axoaxonic synapses than normal. Nevertheless, the presence within transplants of regenerated primary afferent synapses with normal features supports the notion that transplants can support or encourage the formation of relays across regions of damaged spinal cord. Our observation that the axons of donor neurons grow into host sciatic nerve at least raises the possibility that transplants can contribute to reestablishing a damaged segmental reflex arc [58].

The possibility that fetal spinal cord transplants might rescue axotomized neurons that would otherwise die was first confirmed in newborn rats [7]. Rubrospinal neurons were permanently rescued by transplants of embryonic spinal cord, a normal target of rubrospinal axons, but not by hippocampus, suggesting that survival after injury depended on target-specific factors. We have subsequently found that the neurons of Clarke's nucleus are rescued in newborn rats not only by fetal transplants of their normal targets, cerebellum and spinal cord, but also by transplants of embryonic neocortex [24]. The axons of Clarke's nucleus neurons do not normally encounter neocortex, which is therefore an inappropriate target for these neurons. This result suggests that the neurons of Clarke's nucleus can be rescued by several different factors or by a single factor that is produced in several regions of the embryonic CNS. This factor has not yet been identified. It appears not to be produced ubiquitously in the fetal CNS, however, because axotomized Clarke's nucleus neurons die in spite of the presence of embryonic striatum transplants [25]. Embryonic CNS transplants also rescue Clarke's nucleus neurons after axotomy in adult rats [24], suggesting that transplants can contribute to recovery in adult as well as in newborns.

When placed into the spinal cord of newborn rats, embryonic spinal cord transplants function as conduits that stimulate or allow the axons of supraspinal neurons to grow across the site of injury. The axons of

corticospinal neurons [8] and of serotonergic [9] and other brainstem neurons [10] traverse transplants placed in the injured thoracic spinal cord of newborn rats and extend into their normal regions of termination as far caudal as the lower lumbar segments of host spinal cord. In part the greater growth of newborn axons is due to the continued elongation of developing axons that have not reached thoracic levels at the time of transplantation and therefore have not been axotomized. At least some of those axons that reach the lumbar segments, however, were interrupted by the spinal cord lesion and then regenerated [10].

Transplant mediated recovery of function

The idea has been tested that the axons that have traversed the site of spinal cord injury and transplantation alter the development or recovery of locomotor function [40]. Newborn rats that received subtotal thoracic spinal cord injuries and transplants of embryonic spinal cord were examined with a battery of tests and compared to rats that received lesions but no transplant. Rats with transplants performed better than rats with thoracic spinal cord lesions alone. For example, when examined 8–12 weeks postoperatively, rats with transplants crossed a mesh runway more quickly and made fewer errors in foot placement than the group with lesion only. They also recovered more quickly from their errors. The results of this study therefore support the notion that axons that traverse the transplant and grow into caudal host spinal cord are responsible for the improved performance. Because the spinal cord lesion was incomplete, however, other mechanisms for the improved performance are possible. One possibility is that the transplants have changed the response to injury of the residual host spinal cord adjacent to the transplant and allowed axons of supraspinal neurons to grow through host spinal cord rather than through the transplant. The axons of corticospinal neurons are known to grow through regions of newborn spinal cord adjacent to injury [6], and transplants of DRG neurons and Schwann cells have been shown to enhance this response [39]. Therefore, supraspinal axons that have traversed host spinal cord rather than the transplant may account for the improved locomotor function or may have contributed to the improved performance.

Locomotor function is also being evaluated by Howland in newborn cats that received a transplant into the site of a complete spinal cord transection. These experiments complement those in rat because the spinal cord lesion is complete rather than subtotal, locomotor function can be analyzed in greater detail in the cat than in rat, and because the anatomical pathways that account for various types of locomotor performance are better defined in cat than in rat. Three types of locomotion, which are mediated by three different types of spinal systems, have been analyzed (see above; reviewed in [22]): bipedal reflex locomotion, quadrupedal reflex locomotion, and conditioned (voluntary) overground locomotion. In cats with thoracic spinal cord transections that have received a transplant on the day after birth, performance of quadrupedal reflex locomotion will suggest that propriospinal connections have grown across the transplant either directly or via relays; conditioned overground locomotion will suggest the growth of axons with perikarya in the brain.

The locomotor function of 2 cats that received transplants of E26 spinal cord into T12 transections on the day after birth has been examined for periods of 6 weeks and 5 months [29,30]. These cats were compared to 2 cats with transections on the day after birth that did not receive transplants. Both groups developed quadrupedal locomotion in addition to bipedal locomotion, but the group with transplants achieved overground locomotion approximately 6 weeks earlier than the group with transection alone. The preliminary results also indicate that the performance of animals with transplants is superior to those with transection alone in the ability of the animals to support their weight, to maintain postural stability, and to coordinate the movement of forelimbs and hindlimbs. Even in animals with transplants, however, the coordination is only sometimes similar to that of normal cats, and overground locomotion is abnormal. The postural stability of the hindlimbs, for example, is impaired, the step cycle is prolonged, and the normal 1:1 pairing of forelimb and hindlimb step cycles is inconsistent. In both cats that received transplants and were studied behaviorally, histological evaluation revealed transplants to be present.

Preliminary studies have also been carried out to identify the anatomical connections in these cats [30]. One animal that received a transplant of E21 spinal cord has so far been studied. Descending serotonergic and noradrenergic axons whose cell bodies are in the brainstem grow extensively in this transplant and enter host spinal cord caudal to the transplant. Serotonergic axons grow as far caudally as the host L6 segment. Regenerated CGRP-immunoreactive host dorsal roots and substance P-containing processes that arise from multiple sources are also found throughout the transplants, where they are accumulated in some areas that resemble the substantia gelatinosa and superficial dorsal horn of normal spinal cord. These preliminary results indicate that transplants enhance the development of locomotor function in newborn cats with complete spinal cord transections and suggest that this effect is mediated by descending axons that grow into the trans-

plants. Whether these axons alone account for the enhanced locomotor function remains to be determined.

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Anatomical and behavioral outcome after spinal cord contusion injury produced by a displacement controlled impact device

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Using a displacement controlled injury device that has been developed at Ohio State for use with rodents, we have characterized the level of displacement necessary to produce groups of animals with mild vs. moderate residual deficits in locomotor function [1]. These deficits have been assessed using simple behavioral measures (open field ratings, inclined plane performance, grid walking performance, and footprint analysis). Anatomical measures of the lesions have included % cross sectional area spared at the lesion epicenter, lesion volume, and 3-dimensional reconstructions. Using this device, we are able to consistently produce lesions with almost no variability in the recorded displacement and little variability in the force required to produce this degree of displacement. We have also confirmed that our behavioral and anatomical measures are useful for separating groups with mild vs. moderate residual deficits and that there is little variability within groups for these measures as well (with the exception of footprint measures). In addition, there is a very high degree of correlation between the physical descriptors of the injury (displacement and force) and the behavioral and anatomical results, and high degrees of correlation between the behavioral and the anatomical results.

We have tested a few of the compounds shown by some laboratories to improve functional outcome after spinal cord injury [2,3]. These include Nalmefene (NLF), an opiate antagonist with increased activity at kappa receptors, U-50488H (U-50), a specific kappa agonist, YM-14673 (YM), a TRH analogue, Tirilazad mesylate (U-74006F; LAZ), a methylprednisolone analogue, and methylprednisolone sodium succinate (MPSS). The general design of the experiments has been to compare drug-treated groups to injured controls in blind, semi-randomized trials. Injured controls are repeated with each trial. Most of the experiments used a 1.1 mm displacement injury which produces a profound early deficit followed by minimal recovery of locomotion. All positive drug effects have been noted in cases with this level of injury. Improvements in performance in open field testing at 4 weeks have been noted following: NLF (0.1 mg/kg), U-50 (10 mg/kg), and YM (1 mg/kg) all given as an iv bolus at 30 min post-injury; and MPSS (30 and 60 mg/kg) administered immediately and at 2, 4 and 6 h post-injury. Improvements in inclined plane performance were observed after YM administration (same dose as above) and after MPSS (30 mg/kg q/6). YM and MPSS treatment showed increased sparing of fibers (average of 8% and 5% respectively) at the lesion site compared to controls; NLF showed a trend for increased fiber sparing at the lesion site.

Using the current model of the injury device, it is now possible to produce more consistent lesions than in the past. This increased control over the independent variable has significantly improved our ability to detect small, positive therapeutic effects of treatment protocols in rodents. From these data, it also appears that for simple screening studies, open-field and inclined-plane tests are adequate for rats with profound early deficits and minimal recovery of locomotor ability. Grid walking performance is more useful for detecting residual deficits when locomotor ability and hence performance on open field and inclined plane tests are closer to normal. In addition, the simplest measure of anatomical sparing (% cross sectional area spared) was useful for detecting small changes due to drug administration and correlated with the behavioral improvement. (Supported by NS-10165 and NIH training grant NS-07291).

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APPENDIX 19

EXPRESSION OF β-PREPROTACHYKININ mRNA AND TACHYKININS IN RAT DORSAL ROOT GANGLION CELLS FOLLOWING PERIPHERAL OR CENTRAL AXOTOMY

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Abstract—The changes in gene expression and protein synthesis induced in neurons by axotomy usually lead to increased production of axon constituents and decreased production of molecules related to neurotransmission. Exceptions to this generalization occur, however, and it is unclear whether the injury itself changes the pattern of synthesis or whether individual mechanisms regulate the synthesis of the various axonal components. We used *in situ* hybridization histochemistry and immunocytochemistry to compare the changes in L4 and L5 rat dorsal root ganglion neuron levels of preprotachykinin mRNA and tachykinin peptides caused by sciatic nerve injury with those caused by dorsal root injury. Both lesions elicit regeneration, although only the axotomized peripheral processes re-establish functional contact with their targets. In the contralateral, intact dorsal root ganglia approximately 17% of neurons contained detectable levels of both mRNAs and peptides. Sciatic nerve section decreased by 70% the number of neurons labeled for preprotachykinin mRNA at three days post-operatively. Not all cells in the ganglion are axotomized by the sciatic nerve lesion; grain counts over the cells spared by the lesion showed an increased level of labeling, possibly a result of collateral sprouting by these spared cells. By two weeks, the number of cells labeled for preprotachykinin mRNA had decreased to 80% of control levels. The numbers of neurons labeled for tachykinin peptides decreased more slowly and reached approximately 50% of control numbers at two weeks. By six months post-operatively, when regeneration is largely complete, the number of neurons containing both mRNAs and peptides returned to normal. In contrast, dorsal root section did not elicit a decrease in the number of neurons labeled either for the mRNAs or the peptides at any of the post-operative intervals examined.

These results indicate that axotomy is not the stimulus that elicits changes in the expression of genes coding for tachykinins. Evidence is considered indicating that interruption of the supply of peripherally derived nerve growth factor may be responsible for the changes in gene expression for tachykinins after axotomy.

Peripheral nerve lesions alter the proteins synthesized by dorsal root ganglion (DRG) neurons[4,13,14,20,45] in such a way that regeneration of the axotomized peripheral process is supported. Among the changes are increased synthesis of the growth associated protein GAP-43[20,54,59] and the cytoskeletal protein actin[13,40] and decreased content or synthesis of the transmitter-related peptides substance P, somatostatin, cholecystokinin, and calcitonin gene-related peptide.[3,24,31,40,42,52] These changes are consistent with the hypothesis that protein synthesis is modified after axotomy to increase production of molecules necessary for growth of the damaged process and to decrease production of molecules involved in neural transmission.[12] This could imply that a single stimulus, e.g. the injury itself, sets in motion a co-ordinated series of changes in synthesis of molecules which is reversed when regeneration is completed. Exceptions to this generalization have, however, been reported. Synthesis of some axonal constituents, e.g. neurofilament proteins is decreased during regeneration following peripheral nerve injury,[13,43,62] whereas increased content has been reported for peptides which are thought to function as neurotransmitters or neuromodulators, including vasoactive intestinal polypeptide,[31,52,53] histine isoleucine,[31] and galanin.[21] In addition, peripheral nerve injury induces some changes in protein synthesis that axotomy of the centrally directed process does not elicit[14,42,45,46] even though axotomized dorsal root axons regenerate as far as the dorsal root entry zone.[7] DRG neurons do change their levels of synthesis of neurofilament proteins and actin after dorsal rhizotomy[13] although to a lesser extent than after peripheral lesions, but little or no change in synthesis of tubulin[13,43] or in content of the tachykinin substance P is seen.[24] These results suggest that the injury alone does not elicit nor does the initial regenerative response to axotomy require the cascade of metabolic events which appear to be necessary for regeneration. Specific mechanisms

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Abbreviations: DRG, dorsal root ganglion; NGF, nerve growth factor; SSC, sodium chloride 0.15 M/sodium citrate 0.015 M, pH 7; UTP, uridine triphosphate.

may therefore be responsible for the modifications in synthesis of specific proteins or specific classes of proteins.

Although little is known about the molecular mechanisms by which protein synthesis is modified after injury, in at least some cases control is exerted at the level of gene transcription. Either the production or degradation of mRNAs is affected. For example, peripheral nerve lesions increase mRNA levels for some forms of tubulin[20,34] and reduce levels of the mRNA which encodes the neurofilament proteins.[11,19,62] Levels of the substance P mRNA are also reduced for at least the first month after peripheral nerve injury.[40,42] This change is of particular interest because it has been attributed to interruption of the supply of nerve growth factor (NGF),[9] which is derived from the peripheral targets and retrogradely transported to the DRG cell body.[10,55] It is not yet known whether substance P mRNA levels recover when regeneration of the peripheral process is complete. A recent report[42] indicates that injury to the central process did not affect levels of tachykinin mRNA one week post-operatively. Recovery to normal levels after regeneration of the peripheral process with no change after dorsal rhizotomy would be consistent with dependence of substance P mRNA levels on peripherally derived NGF.

To investigate these issues we compared the time course of the changes in the substance P precursor β-preprotachykinin mRNA with the changes in tachykinin peptide content in DRG neurons after section of either the peripheral or the central DRG process. To determine whether mRNA and peptide synthesis were completely or partially reduced in the affected neurons and then recovered, we used *in situ* hybridization and immunocytochemical methods. These techniques permit levels of mRNA and peptide to be assessed in individual neurons.

EXPERIMENTAL PROCEDURES

Surgical procedures

Adult female Sprague–Dawley (Zivic Miller, Allison Park, PA) rats (250–300 g) were anesthetized with chloral hydrate (350 mg/kg, i.p.). The right sciatic nerve of 12 animals was transected in the middle one-third of the thigh, approximately 5.5 cm from the L5 DRG and 6 cm from the L4 DRG. The proximal and distal portions of the nerve were reapposed with a single suture (9–0 silk) through the epineurium, and the wound was closed in layers. These rats survived for 6 h ($n = 1$), one day ($n = 2$), three days ($n = 3$), two weeks ($n = 3$), or more than six months ($n = 3$). The dorsal roots (L3–6) of nine rats were exposed by laminectomies and cut 0.5 cm proximal to the ganglia. The cut ends of the dorsal roots were reapposed and covered with Gelfoam, and the wound was closed. The rats survived for one day ($n = 3$), three days ($n = 3$), or two weeks ($n = 3$). DRGs from three intact rats that had been previously analysed[16] were used for comparison for both experiments.

Tissue preparation

All rats were killed under deep anesthesia (nembutal 65 mg/kg, i.p.) by intracardiac perfusion with 50 ml normal saline, followed by 400 ml freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3). The L4 and L5 DRG were dissected free, placed in 30% sterile sucrose buffer overnight, embedded in mounting medium (Tissue tek, Miles), and kept frozen at −20°C until sectioning. Sections (12 μm) were cut on a cryostat, thaw-mounted on triply subbed slides (porcine gelatin and chromalum), and thoroughly dried at room temperature. Adjacent sections were mounted on two series of slides. One series was stored at 4°C and processed for immunocytochemistry; the other was stored at −70°C and prepared for *in situ* hybridization histochemistry.

Immunocytochemistry

Antiserum to substance P was purchased from INCSTAR (Stillwater, MN). Because the antiserum is directed against the carboxyl-terminus amino acids common to tachykinins, reaction product represents members of the tachykinin family in addition to substance P.

Mounted sections were reacted on slides with substance P antibody at 1:1000 dilution according to the avidin–biotin method of Hsu *et al.*[22,23] The sections were then treated with biotinylated goat anti-rabbit IgG and avidin–biotinylated horseradish peroxidase complex (Vectastain ABC Kit, Burlingham) as specified by the manufacturer. The controls for non-specific staining have been described in detail previously.[56,57]

Synthesis of RNA probe and in situ *hybridization histochemistry*

The cDNA template for β-preprotachykinin was a 345-base pair cDNA containing exons two to six of the gene. The clone cHSPp11, isolated from a human brain library,[1] was obtained from Dr H.-U. Affolter. The specificity of hybridization for this probe has previously been characterized by Northern blot analysis.[1]

The cDNA, inserted into the transcription vector pSP65 (Promega Biotech, Madison, WI), was restricted appropriately, and transcribed according to the manufacturer's specifications using 2.5 μM ([^{35}S]UPT) (1000 Ci/mmol, New England Nuclear) and 10 μM unlabeled UTP, with all other nucleotides unlabeled and present in large excess. The cDNA for preprotachykinin was inserted in either direction with regard to the SP6 promoter, resulting in the synthesis of both a control or sense (identical to the cellular mRNA) and test or antisense (complementary to the cellular mRNA) RNA probes. Specificity of the hybridization has been assessed by verifying that no labeled cells were present in sections processed under the same conditions with the sense probe. In addition, previously we have shown that using the same protocol, cells labeled with the preprotachykinin antisense RNA probe in the DRG also expressed tachykinin-like immunoreactivity.[16]

Hybridization was performed according to the method of Chesselet *et al.*,[8] as previously described.[16] Slide-mounted tissue was rinsed in buffer, acetylated, treated with glycine in Tris–HCl, dehydrated and air-dried. Two to 4 ng/slide of [^{35}S]RNA probe in 25 μl or hybridization mixture [40% formamide, 10% dextran sulfate, 4 × sodium chloride 0.15 M/sodium citrate 0.015 M, pH 7 (4 × SSC) (1 × SSC)] 10 mM dithiothreitol, 1 ng/ml sheared salmon sperm DNA, 1 mg/ml *Escherichia coli* tRNA, 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinyl pyrolidone, 10 mg/ml bovine serum albumin) were placed on each slide. Hybridization was carried out in humid chambers for 3.5 h at 50°C. Post-hybridization procedures included washes in 50% formamide/2 × SSC at 52°C, a 30 min treatment with RNAase A (100 μg/ml, Sigma) at 37°C, and rinses in buffer. Following an overnight wash in 2 × SSC with Triton X-100, slides were rinsed, dehydrated, de-lipidated and air-dried. Autoradiography was carried out using NTB-3 emulsion (Kodak). Slides were placed in light-tight boxes and exposed at 4°C for three to eight weeks, developed (D-19 Kodak), fixed and counterstained with hematoxylin and eosin.

Data collection and analysis

Longitudinal sections of DRG were outlined using camera lucida for analysis of slides prepared both for *in situ* hybridization histochemistry and immunocytochemistry. Every neuronal perikaryon with a visible nucleus within a section of DRG was traced. The circumferences of all neuronal somata were measured, and calculations of the cross-sectional areas were made using the Bioquant planimetry system. Each cell body was scored as either labeled or unlabeled using the following criteria. For *in situ* hybridization histochemistry, a cell was defined as labeled if at least 10 reduced silver grains were present in the emulsion overlying the cytoplasm (background was approximately one grain/cell soma). For immunocytochemistry, labeled cells were easily differentiated from unlabeled cells by the presence of a dense brown immunoreaction product in the cytoplasm. Four randomly selected sections of a DRG were examined for each of three animals at each survival time, resulting in a total mapping of hybridization positive or immunoreactive cells in 12 sections at each experimental time for each experimental condition.

Histograms representing the cross-sectional area of every cell were compiled in order to compare the distribution of the labeled cells with the unlabeled cell populations. The mean cross-sectional areas of the labeled cells were compared with the mean cell areas of the unlabeled DRG population for each of the experimental conditions. Results obtained with *in situ* hybridization histochemistry were compared with those obtained with immunocytochemistry and the ratios between the labeled cell population and the unlabeled cell population were determined. In addition, the proportions obtained for each experimental condition were compared. Differences between mean ratios were analysed by means of Mann–Whitney *U*-tests.

The radioautographic reaction was quantified on DRGs from three animals killed three days after right sciatic nerve lesion. Two sections from the L4 DRG were analysed from control and experimental sides from each animal. All of the tissue analysed by this method was reacted and developed together to reduce the variability in labeling. The relative amount of labeling per cell was determined using a Morphon Image Analysis system.[61] Using this system, the analog video signal from the DRG cell is converted to a digital image, consisting of 512 × 480 pixels, each with a gray value ranging from 0 to 255. A cluster of silver grains associated with a cell was circled, using a mouse; the threshold level was adjusted to distinguish between grains and background staining before each measurement, and the portion of the encircled area above threshold was measured. The correlation between visual grain counts and the area labeled as measured by the Morphon Image Analysis system is highly reliable ($r = 0.95$).[61] An average of three separate measurements from each section was made of tissue adjacent to labeled cells to determine background silver grain density. The mean of these measurements was subtracted from the values obtained from this section. The corrected values for DRG cells from control and experimental sides were analysed using a paired Student's *t*-test.

RESULTS

DRG cells containing β-preprotachykinin mRNA were identified with *in situ* hybridization histochemistry and tachykinin-containing cells were visualized immunocytochemically. On the intact side, following both surgical procedures and at all post-operative survival periods, between 14 and 20% of the total DRG population labeled for β-preprotachykinin mRNA or for tachykinin peptide (Tables 1 and 2).

Table 1. Summary of results following sciatic nerve cut

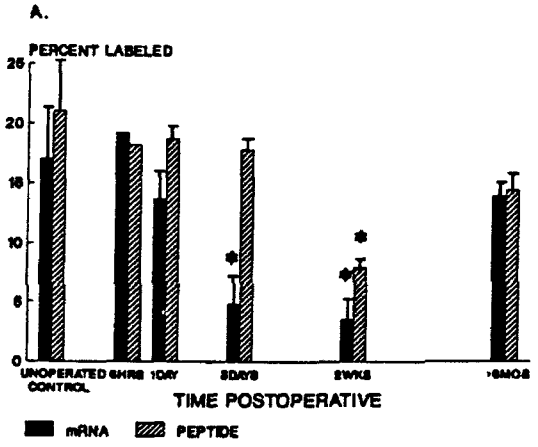
	Six hours ($n = 1$)		One day ($n = 2$)		Three days ($n = 3$)		Two weeks ($n = 3$)		Six months ($n = 3$)	
	Total cells counted	Percentage of cells labeled	Total cells counted	Percentage of cells labeled (+S.E.M.)	Total cells counted	Percentage of cells labeled (+S.E.M.)	Total cells counted	Percentage of cells labeled (+S.E.M.)	Total cells counted	Percentage of cells labeled (+S.E.M.)
In situ hybridization Histochemistry										
Control side	342	16.7	1231	14.3 ± 0.43	1538	16.9 ± 1.00	1227	14.3 ± 0.33	1340	14.7 ± 1.32
Experimental	516	19.2	1206	13.7 ± 1.77	1518	4.8 ± 1.60*	1223	3.5 ± 0.63*	1249	13.9 ± 0.95
Immunocytochemistry										
Control side	532	19.9	1914	16.2 ± 1.12	1724	18.4 ± 1.67	1336	13.6 ± 0.54	1365	14.0 ± 0.32
Experimental	452	18.2	1670	18.7 ± 0.94	1475	17.8 ± 0.92	1223	7.9 ± 0.48*	1388	14.4 ± 0.80

*Significance at the $P < 0.00$ level by Mann–Whitney *U*-test.

Table 2. Summary of results following dorsal root cut

	One day ($n = 3$)		Three days ($n = 3$)		Two weeks ($n = 3$)	
	Total cells counted	Percentage of cells labeled (+S.E.M.)	Total cells counted	Percentage of cells labeled (+S.E.M.)	Total cells counted	Percentage of cells labeled (+S.E.M.)
In situ hybridization						
Histochemistry						
Control side	1272	15.5 ± 0.43	1250	14.8 ± 0.38	1053	15.7 ± 0.74
Experimental	1317	16.9 ± 3.20	1107	15.4 ± 1.37	860	15.7 ± 0.33
Immunocytochemistry						
Control side	1219	16.5 ± 0.73	1200	16.2 ± 1.64	921	13.9 ± 0.90
Experimental	1247	17.8 ± 0.93	1260	17.9 ± 0.33	817	14.9 ± 0.88

These results are comparable with our previous study in normal animals, where neurons containing mRNA for β-preprotachykinin comprised an average of 17% of the total number of somata in each DRG and 20% of the total number of DRG neurons contained the tachykinin peptide.[16]



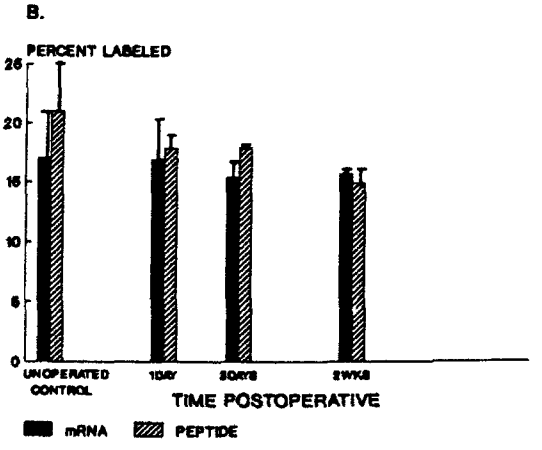


Fig. 1. The percentage of cells labeled by *in situ* hybridization histochemistry for β-preprotachykinin mRNA (black bars) and by immunocytochemistry for the tachykinin peptides (hatched bars) at various times following sciatic nerve cut (A) and dorsal root cut (B). Stars indicate statistical significance at the $P < 0.05$ level by Mann-Whitney U-test.

Sciatic nerve section (Fig. 1A, Table 1)

Six hours following unilateral sciatic nerve section, similar percentages of the total DRG population were labeled for β-preprotachykinin mRNA (19.2%) and for the tachykinin peptide (18.2%) on the axotomized side, and these percentages differed little from those found on the intact, contralateral side.

One day following sciatic nerve section, there was a slight but not statistically significant decrease in the percentage of the total neuronal population that labeled for the message, while the numbers and density of peptide staining showed no such change.

Three days post-operatively only 4.8% of the total DRG population labeled for β-preprotachykinin mRNA on the axotomized side, a decrease of 70% from control values. In adjacent sections of DRG, 17.8% of the cells showed tachykinin immunoreactivity, a number similar to that seen in controls. The percentages of neurons labeled for β-preprotachykinin mRNA and tachykinins are significantly different ($P < 0.05$). In Fig. 2, photomicrographs illustrate the decreased numbers of cells hybridized on the axotomixed side (Fig. 2A), while the numbers of cells labeled by hybridization on the control side (Fig. 2B) and by immunocytochemistry on both the experimental (Fig. 2C) and control (Fig. 2D) sides lie within the normal range.

Since the reduction in the number of cells labeled for preprotachykinin mRNA at three days post-operatively was much greater than the decrease in the amount of mRNA for SP, measured by dot-blot,[40] we also determined labeling density over the DRG cells on control and experimental sides at this time. Labeled DRG cells on the control side did not differ in the mean labeling per cell among animals, nor did the mean labeling per cell on the experimental side differ among animals. The mean labeling per cell on the experimental side was, however, increased by approximately 50% compared with the mean labeling per cell on the control side (mean ± S.E. of the area occupied by silver grains for control DRG cells = 20.5 ± 1.2 and for experimental DRG cells = 33.2 ± 2.1; $P < 0.005$, paired t-test). These results suggest that the DRG cells which continued to express the tachykinin mRNAs after axotomy either

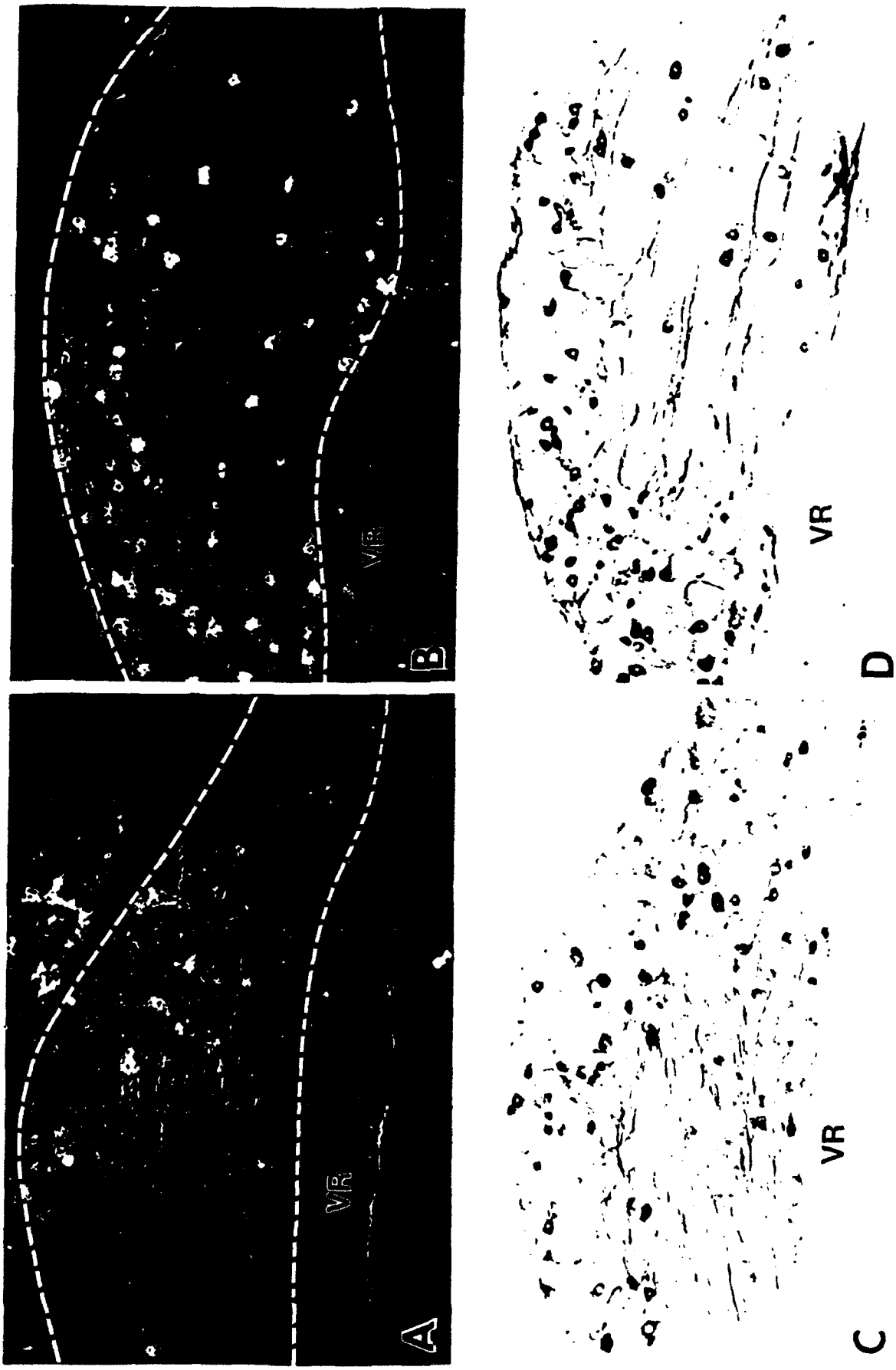


Fig. 2. *In situ* hybridization histochemistry autoradiographs of DRG three days following sciatic nerve cut on the axotomized side (A) and control side (B). Sections were counterstained with hematoxylin and eosin. Light micrographs processed for immunocytochemistry of adjacent sections of the axotomized side (C) and control side (D). Scale bar = 50 μm

increased the production or decreased the rate of turnover of the mRNAs.

Two weeks post-operatively the number of neurons containing β-preprotachykinin mRNA remained decreased; 3.5% of the total DRG population was labeled, a decrease of 80% from control values. At this stage tachykinin content was also decreased, only 7.9% of all cells were immunoreactive, an approximately 50% decrease from control values (Fig. 1A, Table 1). Both ratios are different from control values ($P < 0.05$) and from each other ($P < 0.05$).

Six months following sciatic nerve section, the proportion of cells labeled for the message and for the peptide returned to 85% of control, a level not significantly different from control values.

Dorsal root section (Fig. 1B, Table 2)

One day, three days and two weeks following unilateral central axotomy, equal numbers of cells labeled for the message and for the peptide. In addition at all post-operative survival periods following central axotomy for both mRNA and peptide there was no significant difference between the percentage of labeled cells on control and axotomized sides ($P > 0.05$).

Characteristics of labeled cells

The mean somal areas of all labeled cells were not significantly different from normal at any survival period after either peripheral or central axotomy ($P > 0.05$) and the mean sizes were centered in the smaller range of the distribution of soma sizes for all DRG cells. The mean somal areas of all unlabeled cells for both techniques and for all survival periods following sciatic nerve section and dorsal root section were also comparable ($P > 0.05$) (Fig. 3A,B). Axotomy of either peripheral or central processes does not therefore cause a generalized shrinkage in the mean cell size of the DRG population.

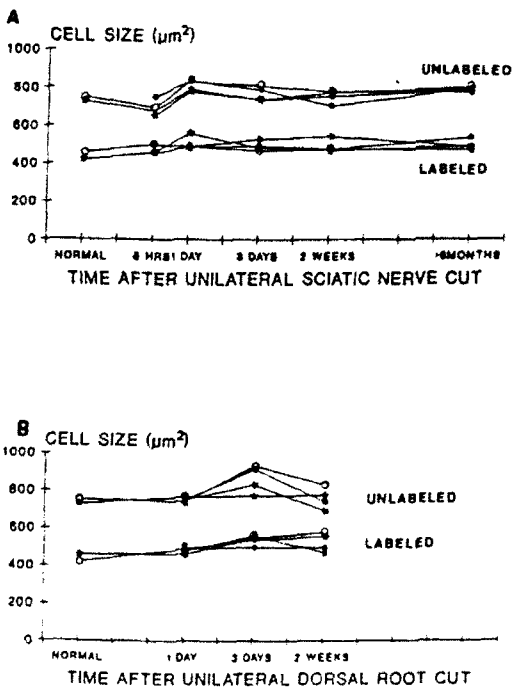


Fig. 3. The mean soma size for labeled and unlabeled populations of DRG neurons using *in situ* hybridization histochemistry (stars) and immunocytochemistry (circles) are presented following sciatic nerve cut (A) and dorsal root cut (B).

DISCUSSION

In the present study we used *in situ* hybridization histochemistry and immunocytochemistry to determine the number of lumbar DRG neurons that synthesize β-preprotachykinin mRNAs and tachykinins after axotomy of their peripheral or central processes. The major findings are that peripheral axotomy decreases mRNA and tachykinin labeling over DRG cells while central axotomy does not. The results from the *in situ* histochemistry studies are in accord with those of Noguchi *et al.*,[42] but we have extended their findings by demonstrating the time course of the effect and the dissociation of the time course of changes in the peptide content, as shown by the immunocytochemical reaction, from the change in synthesis of the peptide.

Control dorsal root ganglion

As in the DRG of normal rats,[16] the DRG contralateral to sciatic nerve or dorsal root section contains between 14 and 20% of neurons that label for tachykinin peptide or preprotachykinin mRNA. Our measurements of the somal areas of these neurons show that most are among the "small dark" DRG neurons that have been distinguished histologically.[21,47] Because we used an antibody raised against the C-terminal amino acids common to tachykinins, we cannot distinguish among the three tachykinins and additional biosynthetic processing products or metabolites that have been described in rat DRG.[32,44,60] Most of the tachykinin immunoreactivity is likely to be substance P, because substance P is approximately twice as abundant as substance K (neurokinin A) in DRG, and levels of neuromedin K (neurokinin B) are extremely low.[44,60] Substance P and substance K are synthesized from the preprotachykinin I gene[37,38] after differential splicing of the primary RNA transcript to produce α-, β- and γ-preprotachykinin mRNAs.[26,28,30] All three of these mRNAs are likely to be detected by the mRNA probe that we used for *in situ* hybridization histochemistry. In rats CNS tissues that express this gene, γ-mRNA represents 80% of the total preprotachykinin mRNA, β-mRNA represents 20%, and α-mRNA less than 1%.[38] Neuromedin K derives from a distinct preprotachykinin gene[6,27] and its mRNA was thus not detected in our study.

Sciatic nerve lesion

Peripheral nerve injury reduces concentrations of substance P in DRG neurons.[3,4,24,58] These reductions are due, at least in part, to pretranslational mechanisms because tachykinin synthesis is even more profoundly reduced than concentrations of substance P.[39] Using dot-blot analysis, Nielsch and Keen[40] found that tachykinin mRNA levels fell to 40% of control values by three days post-operatively in the L5 DRG after sciatic nerve section. Our present results using *in situ* hybridization show that the percentage of L4 and L5 DRG neurons that contain detectable levels of tachykinin mRNA is reduced to 20% of control. Approximately 70–80% of L5 DRG neurons are axotomized by sciatic nerve section in the mid-thigh,[18] the axotomized neurons therefore appear to have ceased completely to synthesize tachykinin mRNA. The remaining 20–30% of the DRG cells are not injured by the sciatic lesion. Our findings taken together with those of Nielsch and Keen would predict that the uninjured tachykinin-containing DRG neurons increase tachykinin mRNA production or reduce mRNA turnover, which is a reasonable possibility since DRG neurons have a considerable reserve capacity to increase tachykinin synthesis.[29,35,41] Measurements of the grain densities over DRG cells three days after sciatic nerve lesions did show a significant increase in labeling compared with control DRG cells on the opposite side. This increased labeling may reflect an increased synthesis of tachykinins associated with collateral sprouting by the 20% of DRG cells spared by the sciatic nerve lesion.

The number of cells containing detectable levels of tachykinin mRNA decreased more rapidly than the number containing peptide and the decrease was more complete. We found that the number of cells containing detectable label for tachykinin mRNA began to be reduced by one day after sciatic nerve cut and that the maximal reduction was observed at three and 14 days. Similar results were reported for substance P mRNA using biochemical methods.[40] Using immunocytochemical methods we found normal numbers of neurons labeled for tachykinin peptide at three days after sciatic nerve injury and a 50% reduction in labeled neurons at two weeks. Studies which have used radioimmunoassay to measure DRG tachykinin levels after sciatic nerve section similarly showed no change at one to two days, significant reductions at four to six days, and 40–50% reductions at 14 days.[5,24] It is likely that levels of tachykinin are reduced more slowly than mRNA levels because the peptides are metabolized more slowly than the mRNAs. In DRG cultured in the absence of NGF, approximately 50% reductions in levels of preprotachykinin mRNA are observed by two to three days in culture, and in substance P levels by four to five days.[29] It is not clear, however, why more neurons continue to be labeled for tachykinin peptide than for mRNA even as long as two weeks after injury. One possibility is that the sensitivity of our immunocytochemical and *in situ* hybridization techniques differs, but at least in normal DRG we have found that the methods label very similar numbers of neurons.[16] More likely explanations are that the degradation products of tachykinin peptides continue to be detected by immunocytochemical methods or that a proportion of tachykinin peptide is metabolized extremely slowly.[15,39] These results do indicate that levels of mRNA are a more sensitive indicator of the changing metabolic state of the neuron than are levels of protein.

At times longer than six months following sciatic nerve section, when regeneration is largely completed, the numbers of neurons labeled for tachykinin mRNA and peptide has recovered to 85% of normal. This number of labeled neurons does not differ statistically from the number present normally, but a modest decrease would also be consistent with the death of a small percentage of axotomized neurons. We have previously found that 20% of all L5 DRG neurons died when the sciatic nerve of adults was cut and ligated to impede regeneration,[18] somewhat less cell death would be anticipated under the conditions of the present study. In both the present and previous studies, we found no change in the mean somal area of DRG neurons, indicating that neither large nor small DRG neurons are particularly vulnerable to axotomy.

Dorsal root lesion

Considerable experimental evidence now suggests that the ability of the DRG neuron to produce both tachykinin peptide[35,51] and mRNA[29] depends on the availability of NGF, which in the intact neuron is supplied to the perikaryon by retrograde transport from the target[10,55] but in the regenerating nerve may also be supplied by Schwann cells.[5,17] Decreased numbers of tachykinin mRNA- and peptide-labeled cells followed by recovery toward normal numbers is consistent with the idea that peripheral nerve section deprives the neuron of NGF and that, when regeneration is complete, normal amounts of NGF are again supplied from the target. We found that the number of cells labeled for tachykinin mRNA or peptide were not affected by section of the dorsal root during the first two post-operative weeks. Thus tachykinin synthesis does not depend on NGF transported from the spinal cord even though the surfaces of the central processes of DRG neurons contain NGF receptors which can take up and transport NGF.[49]

Transmitter synthesis is likely to be regulated by additional mechanisms besides the supply of growth factors. Changing levels of activity, for example, also modify levels of synthesis of transmitters and modulators (e.g. treatment with antagonists and agonists of neurotransmitter receptors,[2] denervation,[48] depolarization,[36,50] peripheral stimulation.[25,35,41] Furthermore, our observation that peripheral axotomy affects

the number of cells labeled for tachykinin mRNA and peptide but central axotomy does not is similar to the results that have been reported for some mRNAs and peptides but contrasts with others. Peripheral but not central axotomy increases synthesis of the peptide or the mRNAs for some forms of tubulin[13,20,34] and GAP-43.[20,54,59] In contrast both peripheral and central lesions increase actin synthesis[13] and decrease synthesis of the neurofilament proteins.[11,13,41,62] The stimuli that affect the synthesis of each of the individual components of the regenerating axon are unknown, but the elimination of growth factors is unlikely to account for the changes in all of these proteins.

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Death of Some Dorsal Root Ganglion Neurons and Plasticity of Others Following Sciatic Nerve Section in Adult and Neonatal Rats

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ABSTRACT

Newborn animals recover from neurological injury to a greater extent than adults in spite of the greater vulnerability of developing neurons to retrograde or transneuronal degeneration (Kennard, '42; Goldman, '74; Prendergast and Stelzner, '76; Bregman and Goldberger, '82, '83). The cellular mechanisms underlying this "infant lesion effect" are incompletely understood (Bregman and Goldberger, '82). The dorsal root ganglion (DRG) is an excellent model in which to compare the developing and adult nervous system with respect to the effects of axotomy on cell survival and cellular function. We studied the survival of L5 DRG neurons after section-ligation of the sciatic nerve of adult and neonatal rats and used qualitative and quantitative immunocytochemical methods to examine changes in intraspinal substance P immunoreactivity (SPIR). Retrograde transport of wheatgerm agglutinin-horseradish (WGA-HRP) peroxidase applied to the sciatic nerve of adult or neonatal rats demonstrated that 70% of the neurons in the normal L5 DRG project into the sciatic nerve at the site of transection. In adults 20% of all L5 DRG neurons died between 10 and 60 days postoperative; in newborns 50% of the neurons died between 5 and 10 days. These results indicate that 30% of axotomized neurons in adults and 75% in neonates die after sciatic nerve section and that neuron loss is both more rapid and more extensive in neonates. No cell death was observed in the L5 DRG of neonates after dorsal rhizotomy, thus suggesting that at this stage of development the survival of DRG neurons depends on the peripheral but not the central process.

SPIR in laminae I and II of both adult and newborn operates decreased and then recovered, but the time course and extent of the recovery differ. In adults SPIR was depleted in the medial portion of the L5 segment ipsilateral to surgery by 10 days postoperative and remained depleted for at least 2 months. By 1 year partial recovery occurred, but remained incomplete even at the longest survival time studied (15 months). SPIR, which is present in the dorsal horn at birth, was diminished in ipsilateral laminae I and II by 4 days after nerve section on the day of birth. Between 30 days and 60 days, the density of SPIR in the dorsal horn ipsilateral to surgery became virtually indistinguishable from that on the contralateral, intact side, suggesting a more rapid and complete recovery than in adults. The area occupied by SPIR staining remained smaller than that seen on the unoperated side, thus suggesting incomplete or abnormal development of the terminal field in the deafferented dorsal horn.

Recutting the sciatic nerve after SPIR recovery depleted the recovered SPIR in both adults and neonates, indicating that in both groups recovery was

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largely due to DRG neurons that had survived initial transection of their axons. The results indicate that in developing animals 25% of the normal numbers of DRG neurons that survive sciatic nerve section can restore the normal pattern and density of the substance P (SP) projection to the dorsal horn. Immature DRG neurons, therefore, appear to be more likely to die after axotomy, yet those that survive are capable of robust anatomical and biochemical reorganization in comparison to axotomized mature DRG cells.

Key words: axotomy, substance P, recovery, immunocytochemistry, computer-assisted image analysis, dorsal horn

Injury of the neurons of young or newborn animals has been shown to produce greater retrograde cell death than that produced by comparable injury in adults (Aldskogius and Risling, '81; Bregman and Goldberger, '83; Yip et al., '84; Schmalbruch, '87). In spite of the greater vulnerability of newborn neurons, however, injury of the immature nervous system is generally followed by greater anatomical reorganization and greater recovery of function than is seen in the adult (Kennard, '42; Goldman, '74; Bregman and Goldberger, '83; Leonard and Goldberger, '87a,b). The cellular and molecular mechanisms that account for this "infant lesion effect" (Bregman and Goldberger, '82) remain incompletely understood.

The dorsal root ganglion (DRG)-spinal cord system provides an accessible and manipulable model for studying two distinct events, the death of some cells and anatomical and biochemical recovery of others. The extent and time course of these two processes are involved in the outcome of nervous system injury in adult and newborn animals. There is general agreement that some DRG neurons die after axotomy of their peripheral processes (Ranson, '06, '09; Hare and Hinsey, '40; Arvidsson et al., '86; however, see Devor and Wall, '78, '81; Sugimoto and Gobel, '82). The extent of neuron death after nerve section in newborns appears to exceed that found after nerve section in adults (Aldskogius and Risling, '81; Yip et al., '84). DRG neurons also alter their pattern of protein synthesis after axotomy (Bisby, '78; Theiler and McClure, '78; Devor and Claman, '80; Perry and Wilson, '81; Tenser, '85; Heumann et al., '87). This results in a decrease in their content of most transmitter-related substances such as substance P (SP) (Jessell et al., '79; Tessler et al., '85; Bisby and Keen, '86; Jones and La Velle, '86). SP in the dorsal horn of the spinal cord is derived principally from a subset of small DRG neurons (Takahashi and Otsuka, '75; Hökfelt et al., '76; Harmar et al., '80; Dodd et al., '83; Price, '85; Henken et al., '87). Some, however, originate in interneurons and supraspinal neurons (Hökfelt et al., '77; Chan-Palay et al., '78; Hökfelt et al., '78; Ljungdahl et al., '78; Seybold, '80; Tessler et al., '81). Retrograde changes in injured DRG cells are demonstrable in regions of the dorsal horn to which these axotomized neurons project, because a decrease in SP immunoreactivity (SPIR) is seen (Jessell et al., '79; Barbut et al., '81; Tessler et al., '85). If regeneration is successful, concentrations of peptides in DRG and dorsal horn return to normal (Bisby and Keen, '86). If regeneration is unsuccessful or prevented, then concentrations of intraspinal peptides will be determined not only by the metabolic capacity of injured DRG cells but also by the ability of those injured DRG neurons to survive axotomy. Surviving axotomized cells and the uninjured cells may display compensatory responses that contribute to recovery (Tessler et al., '85). Similar variables affect the outcome in other areas of the central nervous system (CNS) in which injured axons are unable to regenerate (Aldskogius and Arvidsson, '78; McLoon and La Velle, '81; Bregman and Goldberger, '83).

The consequences of peripheral nerve section in the newborn are likely to differ from those seen in the adult (Jones and La Velle, '86), since both the peripheral and central connections of DRG neurons are functionally immature at birth (Fitzgerald and Swett, '83; Fitzgerald and Gibson, '84; Fitzgerald, '85; Fitzgerald and Vrbova, '85). Not only is sciatic nerve section in newborns likely to produce greater retrograde cell death of DRG neurons than sciatic nerve section in adults, but it is also likely that developing cells will display greater anatomical and biochemical plasticity that will contribute to age-related differences in recovery of SPIR in the spinal cord. When regeneration is impeded, section of the sciatic nerve of adult rats and cats produces a reduction of SPIR within the ipsilateral dorsal horn, which is sustained for at least 2 months (Jessell et al., '79; Barbut et al., '80; Tessler et al., '85). SPIR in the cat dorsal horn later recovers, but the source is unknown (Tessler et al., '85). In the partially denervated cat spinal cord, intact dorsal roots have been shown to be capable of intraspinal axonal sprouting (Goldberger and Murray, '82). Recovery in rats has not been studied, but it might be expected to be less than in cats since rat DRG neurons have been reported to differ from those of cats by being incapable of sprouting (Rodin et al., '83; however, see Polistina et al., '87; Wang et al., '87). The effects of neonatal axotomy might be paradoxical: more cell death and greater plasticity of the surviving DRG neurons.

The present studies were undertaken to determine the association between DRG cell death and intraspinal levels of SP after axotomy in newborn and adult rats. Since it is important to distinguish whether the greater cell death seen in neonates results from a greater vulnerability to axotomy generally or if one axonal process is more vital for DRG neuron survival, we sought to confirm the report that dorsal rhizotomy causes the death of newborn DRG neurons (Yip and Johnson, '84). We then compared the amount of DRG cell death after peripheral nerve section in adult and newborn rats. In addition, we used qualitative and quantitative immunocytochemical methods to assay levels of SPIR in the dorsal horn after section of the adult and newborn sciatic nerve not only to determine whether SPIR recovers in the

dorsal horn and the sour of that recovery, but also to compare the extent and time course of the recovery after injury to the adult and newborn nerve.

MATERIALS AND METHODS

Surgical procedures and tissue preparation

Sprague-Dawley rats (Zivic-Miller Laboratories, Allison Park, PA), 42 adult and 58 neonate, were used in these experiments. Nine adults (60 days–6 months of age; 200–400 g) and 6 newborns (DOB, PO) of either sex served as normal, unoperated controls. Animals were deeply anesthetized with either chloral hydrate (adults, 350 mg/kg i.p.) or hypothermia (neonates) and had the right sciatic nerve transected in the middle one-third of the thigh. In order to impede regeneration, a length of nerve (1 cm in adults and 3 mm in neonates) was resected from the distal stump, and the proximal stump was ligated. The site of sciatic nerve transection was approximately 5.5 cm from the L5 DRG in adults and 1.0 cm in neonates. Rats survived for intervals of 1–450 days after right sciatic nerve section or underwent a second procedure. (1) In order to observe the effects of an acute and chronic lesion in the same animal, 4 adult operates had the left sciatic nerve transected 6–12 months after the right using the same technique. (2) To determine whether axotomized DRG neurons could contribute to recovery of SPIR in the L5 dorsal horn, 4 neonatal and 3 adult operates had the right sciatic nerve recut proximal to the initial lesion 3–10 months later. (3) To determine whether DRG neurons not injured by neonatal axotomy could expand their terminal field into the partially denervated L5 dorsal horn, 4 neonatal operates underwent right femoral nerve resection 6 months after right sciatic nerve lesion. The right femoral nerve was ligated and transected at its emergence from the iliacus and psoas major muscle and a 5-mm length of nerve was resected from the distal stump. Rats subjected to a second procedure survived for an additional 30 days.

In order to determine the degree of DRG cell death following axotomy of their central processes, 6 neonatal (PO) rats underwent unilateral section of the L5 dorsal root at the L5 dorsal root entry zone. Care was taken to ensure that all L5 dorsal root fibers were completely cut. Ventral root fibers were not transected during this procedure. Animals survived for either 6 (N = 3) or 30 days (N = 3).

All rats were sacrificed under deep anesthesia (Nembutal, 50 mg/kg i.p.) by intracardiac perfusion with normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4. Animals were included in the study only if the sciatic nerve showed no evidence of regeneration. At autopsy the site of nerve section was examined under a dissecting microscope to confirm that the ligature was still in place and that axons did not leave the neuroma to reinnervate the distal hindlimb or bridge the gap between proximal and distal stumps. In uncertain cases the proximal stump of the nerve and neuroma were removed, embedded in paraffin, sectioned, and stained with the Bodian method (Bodian, '36) to detect the presence of axons that had grown past the transection. After inspection, spinal segments L1-L6 and S1 as well as the right and left dorsal root ganglia (DRG) from L4-L6 were removed and individually embedded in paraffin. Spinal cord sections to be examined for SPIR were prepared according to the unlabeled antibody method of Sternberger ('86) for examination by light microscopy with methods that have been described previously (Tessler et al., '80, '84). All sections were studied for the presence and distribution of SPIR, and representative samples were photographed.

WGA-HRP labeling of the DRG

In order to determine the percentage of cells in the L5 DRG that project axons into the sciatic nerve at midthigh, the level of transection, a 2% solution of wheatgerm agglutinin-horseradish peroxidase (WGA-HRP) was applied to sciatic nerves at the same level as the transection and used to label L5 DRG cell bodies by retrograde transport. WGA was conjugated to HRP by using the procedure described by Mesulam ('82). The right and left sciatic nerves of one adult rat and the left sciatic nerve of another were studied. The nerve was exposed at midthigh level and crushed with jeweler's forceps. Just distal to the crush, 10 μl of a 2% solution of WGA-HRP was rapidly injected through a Hamilton syringe with a beveled 27-gauge needle. In 5 PO neonates the right sciatic nerve was exposed at the midthigh level and crushed with jeweler's forceps. Just distal to the crush, 1 μl of 2% WGA-HRP was injected through a beveled glass pipet with an outer tip diameter of 0.1 mm. The injection site was closely observed through a dissecting microscope to ensure that the WGA-HRP solution did not leak from the site. The nerve was ligated and cut distal to the injection, immersed in petroleum jelly, and the muscle and skin sutured in layers. After an appropriate survival period (24 hours in neonates, 48 hours in adults), the rats were deeply anesthetized and perfused through the heart with an initial flush of warm saline (40–43°C), which contained 4 ml 30% H_2O_2/liter, followed by 3% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 (4°C). After successive flushes of 10%, 20%, and 30% sucrose in 0.1 M phosphate buffer, pH 7.4, the DRG were dissected out, serially sectioned on a cryostat at 10 μm, and mounted on slides. The WGA-HRP reaction product was visualized with TMB as the chromagen according to the protocol described by Mesulam ('82). Every tenth section was photographed under darkfield illumination, and the photographs were printed at a final magnification of 220× in order to obtain a montage of that section. The coverslips were then removed with xylene, the sections rehydrated in graded ethanol solutions and restained with cresyl violet acetate (Kodak). This procedure removed the TMB reaction product from the DRG neurons and allowed clear visualization of the nuclei of the ganglion cells. The same sections were then rephotographed under brightfield illumination, and photographs were printed again at a final magnification of 220×. Photographs of each section stained for WGA-HRP reaction product and with cresyl violet were then compared. Cells counted as WGA-HRP labeled DRG neurons were those that contained WGA-HRP reaction product on the darkfield photograph and a nucleus on the photograph of the Nissl-stained section. A ratio was then calculated comparing the number of WGA-HRP labeled cells to the total number of cells counted.

DRG cell counts and area

Longitudinal 10-μm serial sections were cut through paraffin-embedded DRG, mounted on slides, and stained with cresyl violet. Neuronal nuclei were counted in every tenth section with the aid of a Leitz Dialux microscope at a final

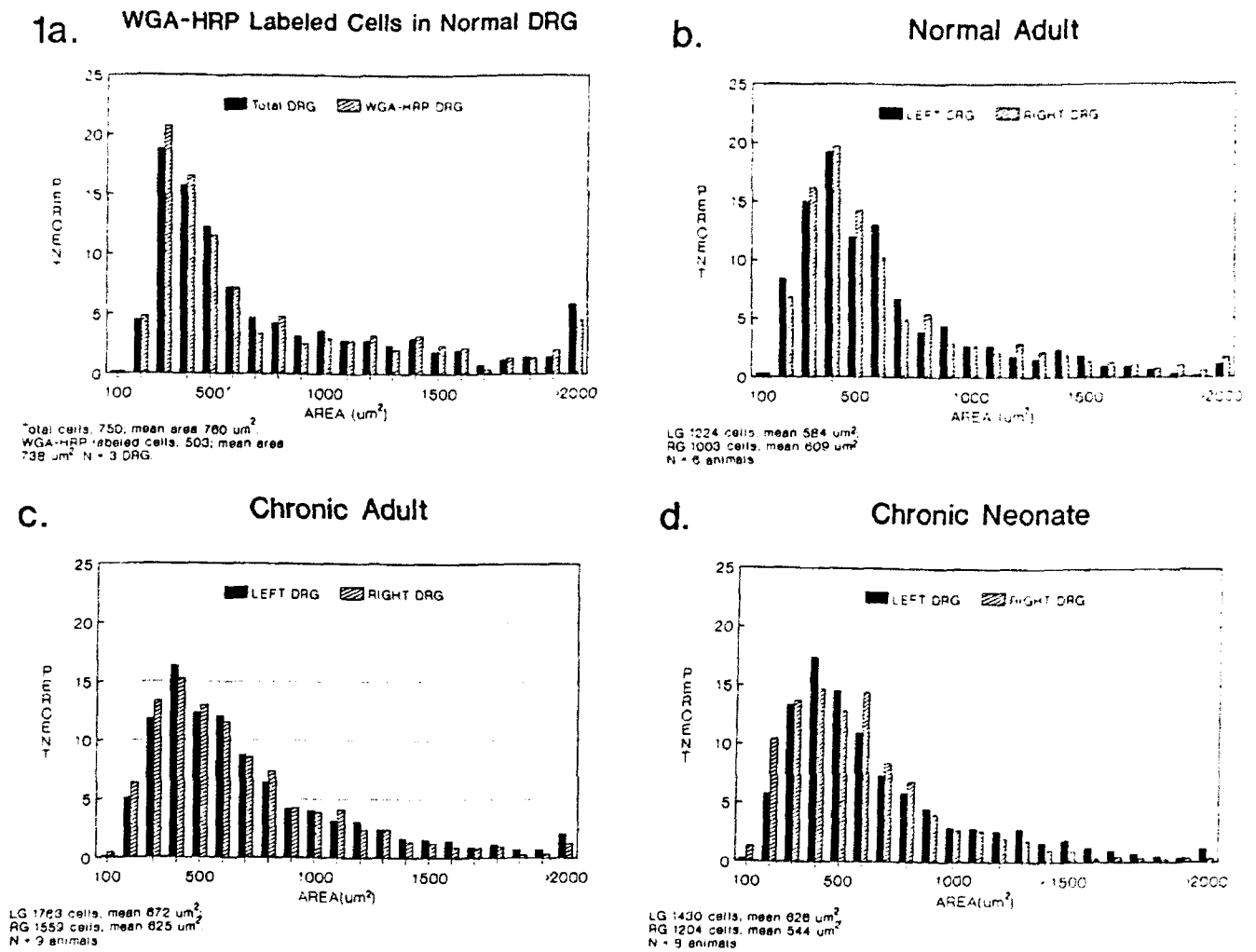

Fig. 1. Cell size histograms showing the distribution of L5 DRG neurons. (a) Histogram comparing the overall cell size population with that of the population of neurons labeled with WGA-HRP. (b–d) Histograms comparing the cell size distribution in the left and right L5 DRG: (b) 6 normal adults, (c) 9 adult animals that had right sciatic nerve section at least 60 days previously, (d) 8 animals that had right sciatic nerve section on the day of birth and survived at least 60 days.

magnification of 510× for animals P10 or older, 816× for animals younger than P10. Average nuclear diameters were determined with planimetric measurements of camera lucida drawings of nuclear cross sections for a minimum of 30 nuclear profiles from each DRG. These data were then processed by means of the Hendry ('76) analysis as modified by Smolen et al. ('83) to obtain a factor for corrected neuron counts. Total neuron number was then determined with this factor.

A cell size analysis was carried out for each ganglion on the basis of planimetric measurements of camera lucida drawings of about 200 perikarya with a nucleus present in the section plane. This sample was generated by measuring random cells in every tenth section throughout the DRG. Size-frequency histograms were generated for each animal and the mean cell size was also computed. A paired comparison t-test was used to look for changes in mean cell size following sciatic nerve section.

Substance P immunocytochemistry

Substance P antiserum (Immunonuclear Corporation, Stillwater, MN) was used at a final dilution of 1:5,000. Sections of spinal cord were labeled in test tubes by the peroxidase-antiperoxidase method of Sternberger ('86). The labeling procedure and controls for nonspecific staining have been described in detail previously (Tessler et al., '80, '84).

Quantitation of SPIR following sciatic nerve section

Measurements to quantitate the area of, and relative amount of SPIR in the dorsal horn gray matter were carried

out with the aid of the Cambridge Instruments Quantimet 920 image analysis system with its detector attached to a Leitz Orthoplan microscope. Brightfield illumination was used with all specimens. Measurements were made at a final magnification of 276×. A minimum of 5 animals was measured at each survival time studied. The area of laminae I and II was outlined in both the right and left dorsal horn of five 15-μm sections selected randomly from the L5 spinal cord segment of each animal. The system was then used to determine for laminae I and II: the combined area of lamina I and II, mean gray level (MGL) of the combined area of lamina I and II as an indication of SPIR intensity, and the area of laminae that contained SPIR. MGL is determined by the system on a gray level scale ranging from absolute black (entire sample area stained) to absolute white (no stain) with 256 interval units. The background in each section of tissue was determined by having the system make a measurement in lamina IX, an area of gray matter having little SPIR. This value was then subtracted from the MGL measurement for the combined area of lamina I and II to yield the value used for analysis. The area of laminae I and II that has staining is that area within the outlined laminae that contains SPIR at a level darker than the background gray level. A right-to-left ratio was then calculated for each of these three measurements for each section and the ratio (R/L) was then used for comparing groups of animals at different survival times. This eliminated the problem of inter-animal variability due to: (1) size differences in the animals due to age or sex and (2) immunocytochemical staining differences associated with fixation and general tissue quality. The mean ratio value for each animal and ultimately for each survival time was calculated for each of the three measurements. A statistical analysis was prepared for both the adult and neonatal operates for each of the three measurements. Overall significance was determined by the Kruskal-Wallis one way ANOVA ($p < 0.05$). If significant differences were present, individual posthoc comparisons where made with the Wilcoxon-Mann-Whitney test corrected for multiple comparisons (Kirk, '68). The statistical analysis was performed with the aid of the Number Cruncher Statistical System (Dr. Jerry L. Hintze, Kaysville UT) program on a Compaq Deskpro IBM compatible personal computer.

RESULTS

WGA-HRP cell counts

The three L5 DRG counted from adults contain very similar numbers of labeled cells (Table 1a). Moreover, the mean cell size and the cell size histogram of WGA-HRP labeled neurons are the same as those of the entire population of neurons in the ganglia, suggesting that retrograde transport of WGA-HRP identifies the full complement of DRG neurons that project into the sciatic nerve (Fig. 1a,b). These results therefore indicate that approximately 70% of the L5 DRG neurons send axons into the sciatic nerve at the level at which we placed our lesions in the adult.

The five L5 DRG counted from PO rats show more inter-animal variability in the number of cells labeled (50–70%, Table 1b). This variability is probably due to the greater difficulty in labeling the sciatic nerve in a newborn rather than a difference in the number of newborn and adult L5 DRG neurons that have axons in the sciatic nerve at this level. Mean cell size measurements and a cell size histogram were not attempted for neonatal animals.

TABLE 1. Number of WGA-HRP Labeled Neurons in L5 Dorsal Root Ganglia

Ganglion	WGA-HRP labeled neurons	Total neurons	Ratio labeled/total
A. Adults			
N42-right	4,108	5,722	0.72
N42-left	3,847	6,109	0.63
N46-left	4,419	6,698	0.66
B. Neonates			
	3,526	7,084	0.50
	3,707	8,084	0.46
	4,059	8,318	0.49
	5,323	8,329	0.64
	4,342	6,429	0.68

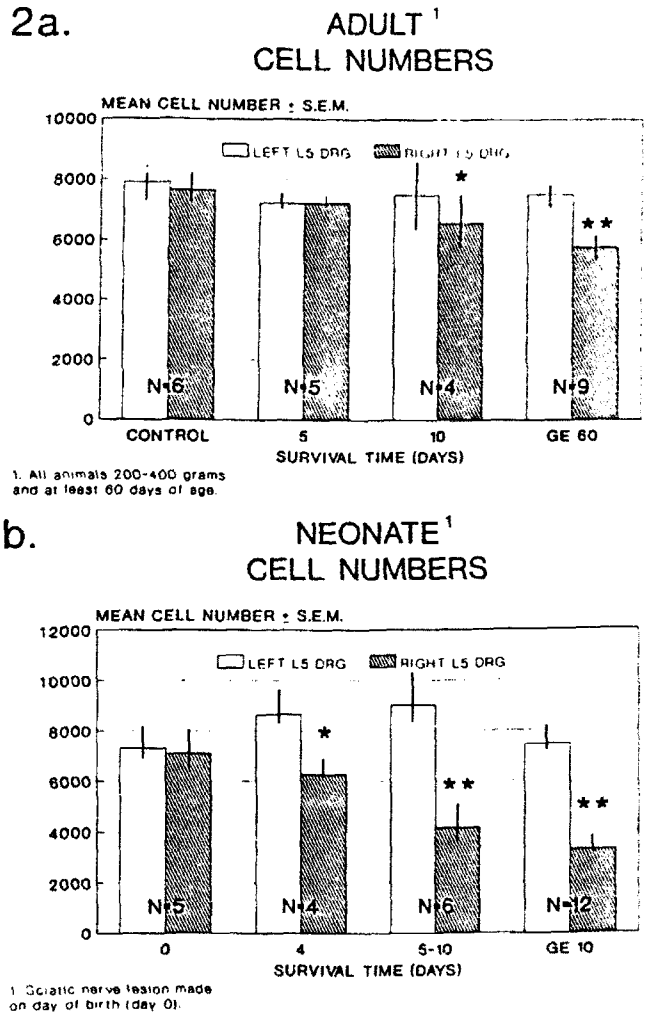

Fig. 2. Bar graph representations of mean cell numbers of the L5 DRG in normal and in operate animals following right sciatic nerve section in (a) adults and (b) neonates. Asterisk(s) indicate(s) a significant right to left difference at $p < 0.05$ (*) or $p < 0.01$ (**) using a paired comparison t-test. Actual values used in these graphs are presented in Tables 2 and 3.

TABLE 2. Number of Neurons in L5 Dorsal Root Ganglia of Adult Operates and Their Mean Cell Area*

Survival time (days)	L5 DRG cell numbers			Mean cell area (μm^2)	
	Left	Right	Ratio R/L	Left	Right
A.					
control	5,502	5,267	0.96	412	451
control	9,459	8,817	0.93	758	511
control	9,038	8,818	0.98	378	573
control	6,792	7,542	1.11	753	975
control	7,697	7,058	0.92	834	669
control	8,898	8,384	0.94	622	603
Mean (N = 6) ± S.E.M.	7,898 ± 571	7,648 ± 508	0.97 ± 0.03	626 ± 72	630 ± 69
B.					
5	7,585	7,094	0.94	637	566
5	8,025	8,060	1.00	468	489
5	7,049	6,954	0.99	979	574
5	6,886	7,073	1.03	639	618
Mean (N = 4) ± S.E.M.	7,386 ± 225	7,295 ± 222	0.99 ± 0.02	681 ± 93	562 ± 27
C.					
10	9,064	7,697	0.85	665	495
10	10,236	8,796	0.86	846	719
10	5,660	5,043	0.89	699	622
10	5,024	4,743	0.94	671	668
Mean (N = 4) ± S.E.M.	7,496 ± 1,102	6,570 ± 862*	0.86 ± 0.02	720 ± 37	626 ± 42
D.					
60	6,210	4,285	0.69	410	448
60	8,299	5,211	0.63	757	576
60	8,707	7,399	0.85	880	795
60	8,075	6,975	0.86	780	798
90	8,931	6,322	0.71	437	338
180	8,627	7,164	0.83	606	635
180	7,333	6,090	0.83	535	541
365	4,920	3,957	0.8	873	809
365	6,507	4,523	0.7	868	851
Mean (N = 9) ± S.E.M.	7,512 ± 431	5,770 ± 413**	0.77 ± 0.03	683 ± 60	643 ± 57

*Right differs significantly from left at the $P < 0.05$ (*) or $P < 0.01$ (**) level using a paired comparison t-test.

L5 DRG cell counts following sciatic nerve section

Adults (Table 2, Fig. 2a). Control animals contain comparable numbers of ganglion cells in their right and left L5 DRG (Table 2a). No change occurs by 5 days after right sciatic nerve section-ligation (Table 2b). By 10 days neuron numbers on the operated side are decreased by approximately 10% compared to the contralateral, intact side (Table 2c), and by 60 days the decrease has reached 20–25% of total DRG cells Table 2d). No further change is observed at survival times up to 365 days postoperative. Since approximately 70% of the L5 DRG neurons have been axotomized, these results indicate that section-ligation of the sciatic nerve in adults causes the death of 30–35% of the axotomized neurons. The mean cell size of neurons on the operated side is not statistically different from neurons on the contralateral, intact side at the postoperative times studied. This result suggests that no subset of adult L5 DRG neurons as determined by size is particularly likely to die after sciatic nerve section (Fig. 1c).

Neonates (Table 3, Fig. 2b). Neonatal control rats contain similar numbers of cells in their right and left L5 DRG on the day of birth (Table 3a) and neuron numbers are comparable to those present in the unoperated L5 DRG of adults (Table 2a). By 3 days after section-ligation of the right sciatic nerve on the day of birth, the operated side shows a decrease in cell numbers of approximately 20–25% compared to the unoperated, control side (Table 3b), and by day 5 the loss is 50% (Table 3c). No additional cell loss is observed at postoperative survival times up to 390 days (Table 3d). This cell loss is significantly greater than that seen after sciatic nerve section in the adult ($p < 0.001$, one-tailed Wilcoxon-Mann-Whitney test). If, as seems likely, similar numbers of L5 DRG contribute axons to the sciatic nerve in newborns and adults (app. 70%), then these results suggest that sciatic nerve section-ligation on the day of birth causes death of 75% of the axotomized neurons. The mean cell size of L5 DRG neurons at survival times longer than 30 days is significantly smaller on the operated side (Fig. 1d). This result could be due to preferential loss of large cells or to failure of all size classes of neurons to develop normally.

Neonatal L5 dorsal root section (Table 4). Section of the L5 dorsal root on PO causes no loss of DRG neurons at either 6 or 30 days following the lesion. This is true regardless of whether the nucleus or the nucleolus (unpublished observations) is counted. This result demonstrates that: (1) axotomy itself is not responsible for the death of DRG neurons after sciatic nerve section in newborns, and (2) the survival of DRG neurons postnatally does not depend on their central processes.

Immunocytochemistry

Sciatic nerve section produces a sequence of changes in SPIR that is similar in the segments that contribute to the nerve (caudal L4-S1). The most consistent changes are observed in laminae I and II ipsilateral to surgery, and these changes are described for L5.

Adults. SPIR is present throughout the entire mediolateral extent of laminae I and II of the normal L5 spinal segment. Two types of reaction product are seen in laminae

TABLE 3. Number of Neurons in L5 Dorsal Root Ganglia of Neonatal Operates and Their Mean Cell Area*

Survival time (days)	L5 DRG cell numbers			Mean cell area (μm^2)	
	Left	Right	Ratio R/L	Left	Right
A.					
0 (control)	8,901	6,983	0.78	100	110
0	6,087	6,095	1.00	99	122
0	6,459	6,533	1.01	97	105
0	6,131	5,691	0.93	121	132
0	9,019	10,234	1.13	116	115
Mean (N = 5) ± S.E.M.	7,319 ± 602	7,107 ± 725	0.97 ± 0.05	107 ± 4	117 ± 4
B.					
4	8,531	6,412	0.75	168	139
4	10,332	7,299	0.71	187	184
4	6,786	5,467	0.81	162	202
4	8,933	5,922	0.66	229	164
Mean (N = 4) ± S.E.M.	8,646 ± 632	6,275 ± 340*	0.73 ± 0.03	187 ± 13	172 ± 12
C.					
5	5,872	2,355	0.4	206	199
5	13,174	7,293	0.55	149	155
5	10,955	4,373	0.4	150	123
10	7,284	3,268	0.45	275	275
10	8,259	5,048	0.61	202	151
10	8,455	2,657	0.31	231	200
Mean (N = 6) ± S.E.M.	9,000 ± 983	4,166 ± 686**	0.45 ± 0.04	236 ± 36	209 ± 25
D.					
30	8,197	3,888	0.47	461	363
30	7,723	2,485	0.32	549	405
30	10,138	4,897	0.48	421	276
30	10,413	4,189	0.40	671	485
60	8,608	4,458	0.52	668	542
60	6,005	2,636	0.44	501	631
60	6,264	3,035	0.48	611	279
90	8,270	1,182	0.14	677	551
120	7,559	1,907	0.25	459	329
210	5,428	3,096	0.57	885	677
365	6,546	2,786	0.43	600	540
390	5,676	4,205	0.74	585	606
Mean (N = 12) ± S.E.M.	7,569 ± 457	3,230 ± 308***	0.44 ± 0.04	591 ± 35	474 ± 39**

*Right differs significantly from left at the $P < 0.01$ (*), $P < 0.005$ (**) or $P < 0.001$ (***) level using a paired comparison t-test.

I and II normally: coarse globular deposits interspersed with finer, punctate granules (Fig. 3a). Sciatic nerve section produces a reduction in SPIR in the medial one-third to one-half of laminae I and II, which is apparent by 5 days postoperatively. SPIR in this region of lamina I and II is maximally depleted by 10 days postoperative. Coarse globular staining is largely eliminated and the little SPIR remaining is mostly finely granular. No further changes in SPIR are seen during the first 60 days of survival (Fig. 3b). During this 10- to 60-day survival time, the amount of SPIR in medial lamina I and II is similar to that seen 10 days following unilateral lumbosacral dorsal rhizotomy in the adult rat (Wang et al., '87).

Survival periods longer than 60 days show a gradual return of SPIR. At 6 months postoperative, SPIR in the medial part of laminae I and II of the operated side continues to be reduced in comparison with the contralateral intact side. However, the amount of punctate reaction product is increased over that observed on the operated side at 10 to 60 days postoperatively. This return of SPIR continues until the longest postoperative survival time studied (450 days, Fig. 3c), but even at this time the amount of reaction product remains less than normal. The restoration of SPIR becomes more apparent when results of a chronic (365 days) sciatic nerve section are compared to an acute sciatic nerve section (30 days) in the same animal (Fig. 3e,f). In this preparation the amount of SPIR present in the medial half of the chronically lesioned dorsal horn clearly exceeds that seen in the contralateral dorsal horn after 30 days.

TABLE 4. Number of Neurons in L5 DRG After Cutting the Right L5 Dorsal Root on the Day of Birth

Survival time (days)	L5 DRG cell numbers		Ratio R/L
	Left	Right	
6	7,528	9,089	1.21
6	6,288	5,908	0.94
6	9,173	8,863	0.97
30	6,795	9,293	1.37
30	8,102	7,183	0.89
30	6,776	6,520	0.96

In order to determine the source of the recovered SP staining, a group of 3 adult rats had the right sciatic nerve recut 90–300 days after the initial section-ligation, and staining was examined 60 days after the second lesion. SPIR in medial laminae I and II of these animals is comparable to that seen after a single sciatic nerve section with 60-day survival (compare Fig. 3b and d). These results suggest that DRG neurons whose axons project into the sciatic nerve and survive axotomy are the principal source for the recovery of SPIR in the dorsal horn.

In summary, section-ligation of the sciatic nerve of adult rats depletes SPIR in the medial portions of laminae I and II, and this depletion is followed by a protracted, incomplete restoration of staining, which is primarily attributable to the DRG neurons with axons in the sciatic nerve. The restored staining is mostly finely granular in nature, but

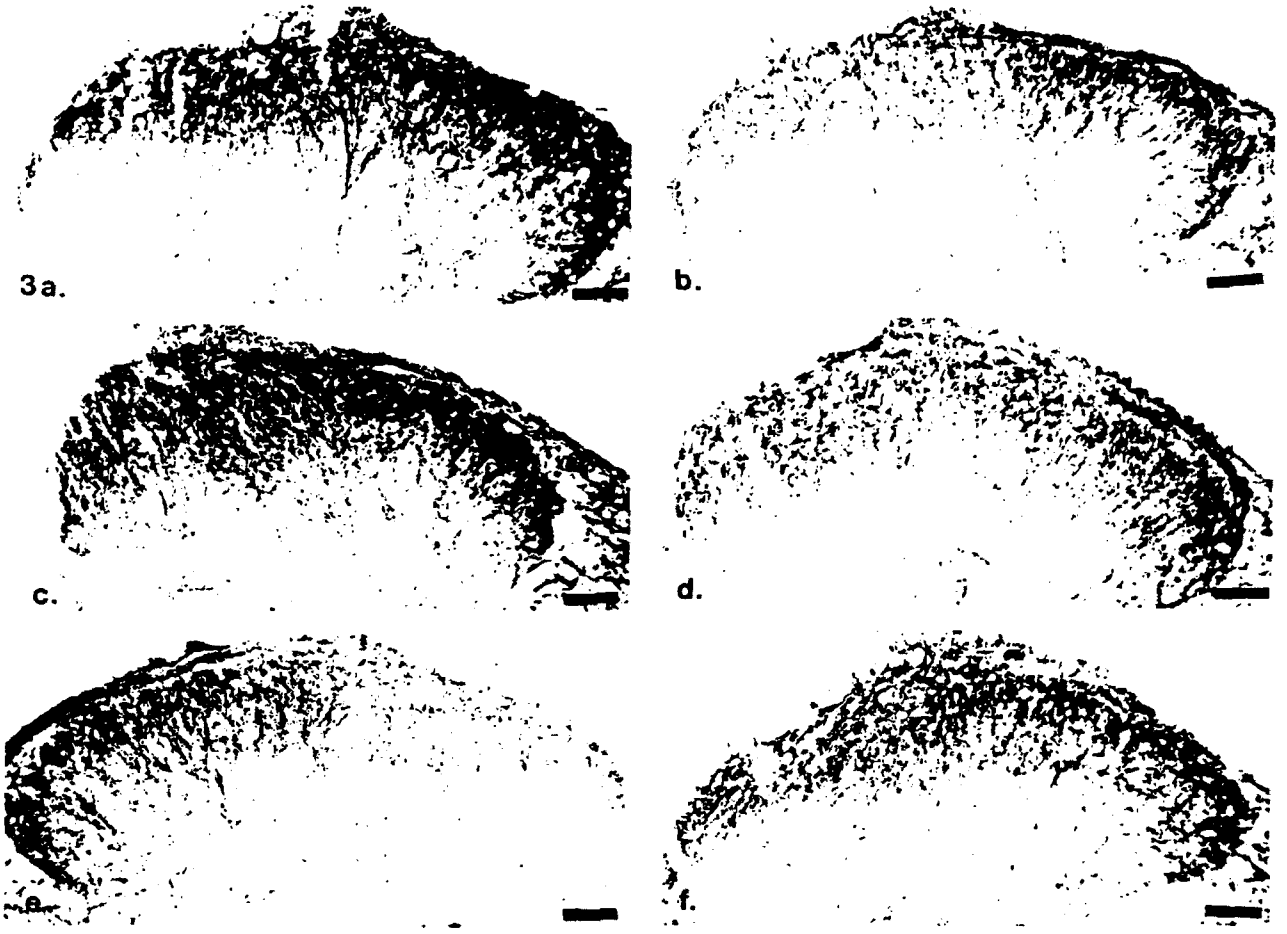

Fig. 3. Photomicrographs showing SPIR in adult animals following right sciatic nerve section. (**a**) Normal right L5 dorsal horn. (**b**) Right L5 dorsal horn 60 days postoperative. (**c**) Right L5 dorsal horn 15 months postoperative. (**d**) Right L5 dorsal horn of a chronic (1 year) animal that then had its right sciatic nerve recut and survived an additional 30 days. (**e,f**) Left and right L5 dorsal horn of the same animal showing SPIR after acute (30 days (**e**)) and chronic (365 days (**f**)) sciatic nerve section in adults. Bar in all figures represents 100 μm.

there is also a return of some coarse globular reaction product.

Neonates. The normal adult distribution of SPIR is present in the L5 segment on the day of birth (P0). Staining extends over the entire width of laminae I and II and consists of finely granular reaction product and is less than in adult spinal cord (Fig. 4a,b). By P13 (14 days postoperative), the amounts as well as the distribution of SPIR in laminae I and II are similar to those of the adult (Fig. 4e). A similar time course of development has been reported previously (Pickel et al., '82; Senba et al., '82). Sciatic nerve section on the day of birth (P0) causes an ipsilateral reduction in SPIR by P3 (4 days postoperative) in the medial half of laminae I and II (Fig. 4c,d). By P13 the reduction in SP staining is less apparent than at P3 (Fig. 4e,f), and the restoration of staining continues at P30. By P60 both the amount and distribution of SPIR in laminae I and II approximate that of the contralateral, intact side, and a relatively normal pattern of staining persists unchanged at survival times up to 390 days, the longest survival time studied (Fig. 4g,h). Coarse globular and finely granular reaction product are both observed. Neonatal operates, therefore, show a much more dense recovery of SPIR than that seen in adult operates.

In order to determine the source of the recovered SP staining, two groups of rats underwent right sciatic nerve section on the day of birth and 365 days later either had the right femoral nerve cut and ligated (N = 4) or the right sciatic nerve cut again (N = 4). Thirty days after femoral nerve section, SP staining in lamina I and II of the L5 segment is unaffected and resembles that seen in the dorsal horn of long-term survivors of neonatal sciatic nerve section (Fig. 4i). Thirty days after the second section of the sciatic nerve, SPIR is largely depleted in the medial half of lamina I and II in the L5 segment, and staining is comparable to that seen 10 to 60 days after sciatic nerve section in the adult (Fig. 4j). These results indicate that DRG neurons that contribute to the sciatic nerve are largely responsible for the recovery of SPIR observed after sciatic nerve section in newborns, but that little if any recovery is due to DRG neurons that contribute to the femoral nerve.

In summary, sciatic nerve section on the day of birth reduces SP staining in the ipsilateral dorsal horn at 4 and 10 days postoperative, and this reduction is followed by a rapid

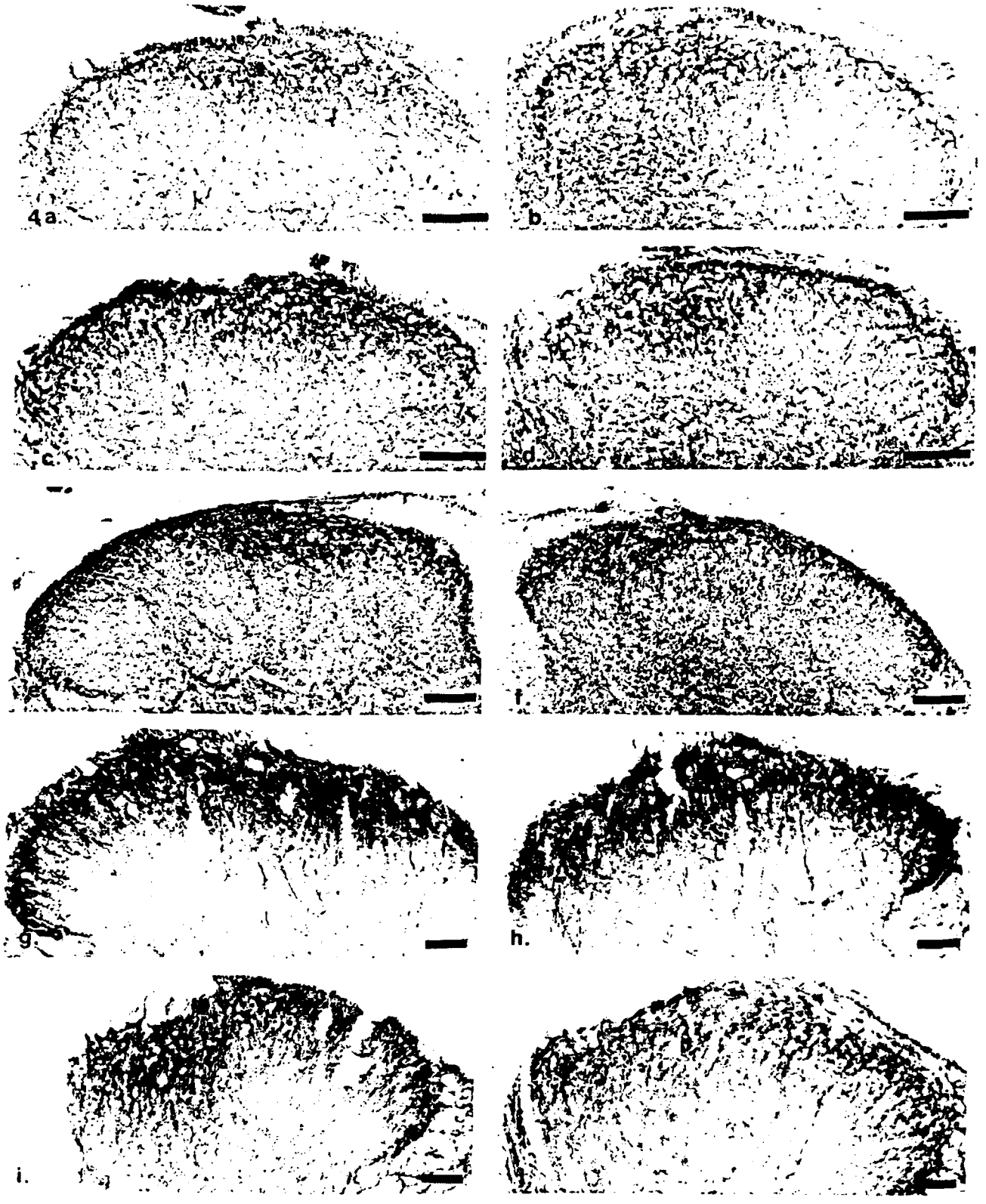

Fig. 4. (**a**,**b**) Photomicrographs showing SPIR in the left and right L5 dorsal horn of an animal on the day of birth (D0). (**c**–**h**) SPIR in the left and right L5 dorsal horns of animals 4 days (c,d), 14 days (e,f) and 390 days (g,h) following right sciatic nerve lesion on D0. (**i**,**j**) SPIR in the right L5 dorsal horn of chronic (365 days) neonatal operates 30 days following either section of the (i) right femoral or (j) resection of the right sciatic nerve. Bar in all figures represents 100 μm.

TABLE 5. Quantitative Analysis of SPIR After Sciatic Nerve Section in Adult Rats[a]

Group	Survival time (days)	Area of Lam. I-II (μm²)	MGL	Area of staining (μm²)
1	control	1.135	1.015	1.105
	control	1.112	0.963	1.012
	control	0.982	1.037	1.011
	control	0.989	1.065	1.079
	control	1.055	0.926	1.089
	control	0.844	1.028	0.958
	control	1.130	0.985	1.105
	control	1.118	0.936	1.034
	control	0.985	0.903	1.229
N = 9	Mean ± S.E.M.	1.039 ± 0.032	0.984 ± 0.019	1.041 ± 0.014
2	10	1.029	0.640	0.190
	10	0.935	0.738	0.412
	10	0.893	0.746	0.449
	10	0.813	0.785	0.383
	10	1.014	0.755	0.364
N = 5	Mean ± S.E.M.	0.937 ± 0.040	0.733 ± 0.025	0.358 ± 0.047
3	30	0.811	0.678	0.306
	30	0.711	0.755	0.326
	60	0.962	0.738	0.488
	60	1.049	0.724	0.425
	60	0.953	0.681	0.346
	60	0.995	0.859	0.749
N = 6	Mean ± S.E.M.	0.914 ± 0.052	0.739 ± 0.027	0.440 ± 0.068
4	90	0.829	0.853	0.610
	180	0.790	0.833	0.536
	180	0.941	0.818	0.505
	270	1.085	0.720	0.370
	365	0.994	1.070	1.047
	365	0.894	0.793	0.532
	450	0.913	1.002	0.871
	450	0.913	0.866	0.897
	450	0.899	0.823	0.620
N = 9	Mean ± S.E.M.	0.918 ± 0.029	0.864 ± 0.036	0.665 ± 0.074

Significant differences among groups[b]
Area Lamina I + II: NONE
MGL: 2, 3 < 1; 2 < 4
Area Staining: 2, 3, 4, < 1; 2 < 4

[a]Values are the ratio of right laminae I and II to left laminae I and II.
[b]Overall significance determined by Kruskal-Wallis one-way ANOVA ($p < 0.05$), individual posthoc comparisons are with Wilcoxon-Mann-Whitney test corrected for multiple comparisons ($p < 0.0083$).

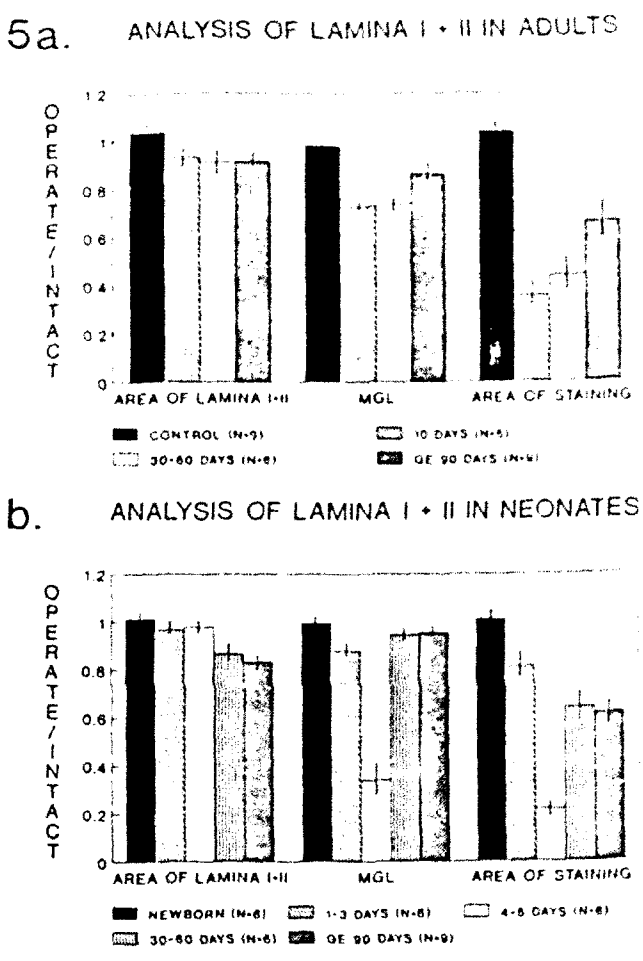

Fig. 5. (a,b) Bar graph representations of the quantitative measurements for L5 laminae I and II in adult (a) and neonatal (b) operates. The values represented in these graphs are presented in Tables 5 and 6.

increase in staining, which by 30 to 60 days is comparable to that seen in the intact contralateral side. As in the adult, DRG neurons that contribute to the sciatic nerve are a major source of the recovery, and even though there are fewer such neurons in the neonatal operate, the restoration of SPIR to the dorsal horn appears similar to that seen in a normal dorsal horn.

QUANTITATIVE ANALYSIS
Adults (Table 5, Fig. 5a)

Area of laminae I and II. Sciatic nerve section-ligation in adults produces no detectable shrinkage of laminae I and II at any of the survival times studied.

Mean gray level. At 10 days postoperative, the intensity of SPIR in the right (operated) laminae I and II is reduced 25% compared to the unoperated left lamina I and II (Table 5, group 2). SPIR remains reduced up to 60 days postoperative (Table 5, group 3). At longer survival times (longer than 90 days), there is a significant increase in the intensity of staining in the right laminae I and II compared to that seen at 10 days, but this intensity remains reduced by 12% compared to normal (Table 5, group 4).

Area of staining. In the L5 lamina I and II of adult operates, the area of staining in right lamina I and II is reduced 65% 10 days postoperative when compared to the left lamina I and II and remains reduced at 60 days (Table 5, groups 2, 3). In the chronic survival group, the area of staining in lamina I and II has doubled compared to that seen in lamina I and II at 10 days (greater than 90 days, Table 5, group 4). However, even at the longest survival times studied, the area of staining remains 32% smaller than in lamina I and II of the unoperated side. These results using a quantitative method confirm that section of the sciatic nerve of adult rats causes an initial decrease in staining intensity and area of staining followed by recovery that is incomplete (Fig. 5a).

Neonates (Table 6, Fig. 5b)

Area of laminae I and II. Following right sciatic nerve section on the day of birth, lamina I and II of the ipsilateral dorsal horn do not develop normally. This is not apparent in the first week following the lesion but 30–60 days following neonatal lesion the ratio of right to left area of lamina I and II is reduced by 13% (Table 6, group 4). This ratio does not change in more chronic preparations (Table 6, group 5) when compared to the ratio for normal animals. This result suggests that L5 lamina I and II of the dorsal

TABLE 6. Quantitative Analysis of SPIR in the L5 Spinal Cord After Neonatal Sciatic Nerve Lesion

Group	Survival time (days)	Area of Lam I-II (μm^2)	MGL	Area of staining (μm^2)
1	0 (control)	1.061	0.996	1.079
	0	0.993	1.001	0.882
	0	0.988	0.912	1.099
	0	0.976	0.933	0.993
	0	0.965	1.076	1.012
	0	1.103	0.968	1.011
N = 6	Mean ± S.E.M.	1.014 ± 0.022	0.995 ± 0.019	1.013 ± 0.031
2	1	1.005	0.889	0.746
	1	0.975	0.782	0.833
	1	1.010	0.875	1.016
	3	1.024	0.988	0.865
	3	0.903	0.826	0.821
	3	0.888	0.928	0.612
N = 6	Mean ± S.E.M.	0.968 ± 0.024	0.881 ± 0.030	0.816 ± 0.055
3	4	0.980	0.597	0.27
	4	0.954	0.415	0.301
	4	1.037	0.138	0.149
	5	0.927	0.393	0.205
	5	0.923	0.259	0.145
	5	1.075	0.238	0.246
N = 6	Mean ± S.E.M.	0.979 ± 0.026	0.340 ± 0.066	0.219 ± 0.026
4	30	0.961	0.858	0.388
	30	0.938	0.972	0.686
	30	0.715	1.023	0.678
	30	0.868	0.962	0.645
	60	0.864	0.946	0.846
	60	0.859	0.914	0.623
N = 6	Mean ± S.E.M.	0.868 ± 0.035	0.946 ± 0.023	0.644 ± 0.060
5	90	0.778	1.052	0.939
	120	0.906	0.869	0.478
	210	0.848	0.980	0.568
	270	0.766	0.969	0.507
	270	0.872	0.924	0.651
	365	0.736	1.005	0.665
	365	0.789	0.932	0.485
	365	0.807	0.975	0.630
	390	1.003	0.897	0.671
N = 9	Mean ± S.E.M.	0.834 ± 0.028	0.956 ± 0.019	0.622 ± 0.047

*Significant differences among groups**
Area Lamina I + II: 4, 5 < 1; 5 < 2, 3
MGL: 3 < 1, 2, 4, 5
Area Staining: 3 < 1, 2, 4, 5; 4, 5 < 1

*Overall significance determined by Kruskal-Wallis one-way ANOVA ($p < 0.05$); individual posthoc comparisons are with Wilcoxon-Mann-Whitney test corrected for multiple comparisons ($p < 0.005$).

horn, when temporarily deafferented by sciatic nerve section on the day of birth, remain permanently atrophied even though there is subsequent reinnervation.

Mean gray level. Ratios obtained from MGL measurements show the intensity of staining to be decreased by 65% in the right laminae I and II 4–5 days following ipsilateral sciatic nerve section on the day of birth (Table 6, group 3). The staining intensity returns toward normal at longer survival times, and after 30 days postoperative the MGL is the same as that seen in normal animals (Table 6, groups 4, 5). This neonatal recovery differs from that seen in the adult in both its rapidity and completeness.

Area of staining. The area of staining in lamina I and II of the operated dorsal horn at 4 days postoperative is reduced 79%. The area of staining rapidly recovers and by 30–60 days it has doubled compared to that present at 4 days but remains reduced by 38% compared to the normal side. Incomplete recovery of staining area at least in part reflects the fact that sciatic nerve section in neonates results in a lamina I and II 16% smaller than normal, which limits the area available for staining.

Taken together, these quantitative results indicate that the recovery of SPIR that follows sciatic nerve section in adults is incomplete both in intensity and area, whereas SPIR recovery after nerve section in neonates fills lamina I and II with a very dense reaction product more like that seen in the normal lamina I and II. However, in the neonate the area covered by staining is the same as that following chronic adult sciatic nerve lesion.

DISCUSSION

The results of the present study show that sciatic nerve section produces different effects on mature and developing DRG neurons. Whereas the neonate shows a much greater loss of DRG neurons than the adult, those DRG neurons that survive are capable of mediating a more rapid and more complete replacement of SPIR in the dorsal horn. The DRG-spinal cord system therefore exhibits characteristics of the "infant lesion effect," in which developing neurons exhibit both greater vulnerability and greater capacity for anatomical and/or biochemical reorganization than mature neurons injured in the same way (Bregman and Goldberger, '82). Because of the ease of access to the DRG and its processes, the mechanisms that account for either cell death or the capacity of neurons to recover after axotomy can be approached more directly than is possible in other areas of the CNS in which the consequences of injury in adulthood and infancy have been compared.

The cell counts in combination with the results of the retrograde WGA-HRP labeling experiment indicate that in the adult 30–35% of the axotomized L5 DRG neurons die and that the period of cell death is extended, occurring 10 to 60 days following injury. These results are consistent with other studies that have used similar techniques (Cavanaugh, '51; Arvidsson et al., '86). In addition the results of our cell size analysis supports the suggestion that no specific size class of DRG neuron is more likely to die after peripheral nerve lesion in adults (Cavanaugh, '51; Risling et al., '83).

Why some DRG neurons live and others die after axotomy is also not understood. One possibility amenable to experimental testing is that survival depends on the existence or development of collateral axons in the peripheral nervous system. There is now a considerable body of evidence that the central and peripheral processes of DRG neurons may branch (reviewed by Coggeshall, '86), so that an individual DRG neuron may have an axon collateral in at least two different peripheral targets (Bahr, '81; Pierau, '82, '84; Taylor, '82), although the number of normal DRG neurons with more than one peripheral branch is controversial (Devor et al., '84). DRG neurons may survive axotomy because they can be maintained by a peripheral process not affected by sciatic nerve lesion or they may be able to extend processes into an abnormal target after injury. Trigeminal ganglion neurons whose axons were transected in the infraorbital nerve on the day of birth have been shown to send aberrant processes into nerves other than the infraorbital (Rhoades et al., '87). These additional axons may, therefore, serve as sustaining collaterals that provide adequate support for survival after axotomy. Another possibility that has yet to be examined is that the subclasses of DRG neurons that have been defined on the basis of immunocytochemical, morphological, or physiological properties differ in their capacity to survive axotomy (Dodd et al., '83; Lawson and Harper, '85; Price, '85). It has been suggested that a subpopulation of DRG neurons remain in an immature form

even in the adult and that this state of immaturity gives these neurons an advantage when reacting to axotomy (Fulton, '87; Mendell et al., '87). These two possibilities could be related if subclasses of DRG neurons, based on their relative maturity, differ in the number of their collateral axons or their ability to establish collaterals after injury.

Neuron loss is greater (75% of the axotomized L5 DRG neurons) and more rapid (5 days postoperatively) when sciatic nerve section is performed within 24 hours of birth. Losses of similar magnitude have been reported previously for neonatal rats (Yip et al., '84; Schmalbruch, '87) and neonatal cats (Aldskogius and Risling, '81). This difference in the magnitude of cell death is related to the age and developmental state of the DRG neurons at the time of axotomy and not to a difference in the number of DRG neurons axotomized, since the results of our WGA-HRP labeling of the neonatal sciatic nerve show that on the day of birth approximately the same proportion of L5 DRG neurons have axons in the sciatic nerve as is found in adults. Our cell size analysis indicates that, although there is a decrease in mean cell size for the population of L5 DRG neurons that survive axotomy on the day of birth, no specific segment of the population appears particularly likely either to survive or to die. Heath et al. ('86) observed no change in the cell size distribution after forelimb amputation in neonates, whereas others have observed a shift in the size spectrum of the cell body (reviewed in Aldskogius et al., '85).

We have also shown that this cell death is related to sectioning the peripheral processes of DRG neurons rather than to a nonspecific effect of axotomy, since cutting the L5 dorsal root on the day of birth has no effect on DRG neuron survival (present study; Bregman, '88, personal communication of unpublished results). We are at present unable to account for the discrepancy between this result and the results of previously published work showing that 50% of L5 DRG neurons die as a result of neonatal dorsal root section (Yip and Johnson, '84).

It is possible that the greater vulnerability of immature neurons reflects greater dependence for survival on substances produced by the targets of these neurons together with an inability of Schwann cells or other supporting cells along the course of the nerve to replace or substitute for substances derived from the targets. Embryonic DRG neurons are known to pass through stages in which their survival depends on nerve growth factor (NGF) (Kessler and Black, '80; Otten et al., '80) and brain-derived neurotrophic factor (BDNF) (Lindsay et al., '85; Davies et al., '86; Kalcheim et al., '87). Experiments using NGF antiserum have suggested that approximately 20% of DRG neurons continue to depend on NGF during the early postnatal period (Yip et al., '84). Additional target-derived substances may be required for the survival of newborn DRG neurons, but these have not yet been identified. It has also not been established that trophic factors are required for the survival of mature DRG neurons (Lindsay, '88).

Our results using qualitative and quantitative immunocytochemical techniques show that SPIR returns to the dorsal horn following sciatic nerve section in both newborns and adults, but that the recovery is more rapid and more complete when the sciatic nerve is cut in newborns. The assumption that the amount of DAB reaction product deposited in the dorsal horn is proportional to the amount of SPIR is supported by previous results (Tessler et al., '85; Micevych et al., '86) indicating that changes in SPIR immunocytochemistry reliably correlate with those determined by radioimmunoassay. The recovery after both adult and newborn injury can be attributed to DRG neurons that contribute to the sciatic nerve and survive axotomy since recutting the sciatic nerve again depletes SPIR in the L5 dorsal horn. It, therefore, appears that the 75% of L5 DRG neurons that survive axotomy of the adult nerve and the remaining 25% of the L5 DRG neurons that survived axotomy in newborns are able to mediate recovery of SPIR in the L5 dorsal horn. Restoration of SPIR in the trigeminal subnucleus caudalis has also been shown to be due to surviving primary afferents (Rhoades et al., '88). Flouride resistant acid phosphatase (FRAP), which is derived from an almost entirely separate population of DRG neurons than SP (Nagy and Hunt, '82; Dalsgaard et al, '84), also recovers after adult or neonatal sciatic nerve lesion (Devor and Claman, '80; Fitzgerald and Vrbova, '85). Recovery of FRAP in the adult, like SP, depends on DRG neurons that contribute to the sciatic nerve (Devor and Claman, '80). However, the source of recovered SPIR and FRAP after newborn sciatic nerve lesion may differ since, in the neonate, the restored FRAP staining was not abolished by resectioning the sciatic nerve and, therefore, was attributed to axonal sprouting by the intraspinal terminals of adjacent peripheral nerves (Fitzgerald and Vrbova, '85).

The atrophy of the dorsal horn that we observed in neonatal operates and sprouting by primary afferent or intraspinal neurons other than those that contain SP could account for our observation that the area of laminae I and II is decreased and, therefore, the area of SPIR could not recover completely. Our methods do not permit us to exclude a minor contribution from additional sources, which could include not only the expansion of terminal fields of adjacent peripheral nerves but also sprouting of neurons intrinsic to the spinal cord (Tessler et al., '81). WGA-HRP labeling has suggested that saphenous nerve terminals undergo central sprouting in L2–L4 spinal segments following neonatal and adult sciatic nerve section, but the long distance sprouting that would be required to extend into the L5 segment was not observed in our experiments or those of others (Fitzgerald, '85; Kapadia et al., '87). Therefore, it seems that any expansion or reorganization of terminal fields following peripheral axotomy is limited to areas closely adjoining intact terminal fields and in this respect resembles the dorsal root sprouting that has been observed in the spinal cord of adult animals after dorsal rhizotomy (Liu and Chambers, '58; Tessler et al., '80; Goldberger and Murray, '82) or spinal cord hemisection (Murray and Goldberger, '74). Long-distance sprouting after peripheral nerve injuries might be expected to produce extensive changes in receptive fields of neurons within the CNS, but such changes have not been observed in the adult cat spinal cord after sciatic nerve injury (Devor and Wall, '81) or in the rat somatosensory cortex after adult or neonatal sciatic nerve section (Kaas et al., '83; Wall and Cusick, '84, '86). Wall and Cusick ('86) showed less expansion of the saphenous nerve representation in the rat somatosensory cortex after neonatal sciatic nerve section than after the same lesion in adults. The atrophy that appears in the dorsal horn following sciatic nerve section is also present in somatosensory cortex, thereby limiting changes in receptive fields (Wall and Cusick, '86). It, therefore, appears that during the maturation of this somatosensory pathway, a sufficient number of synaptic contacts must form in order to prevent atrophy and perhaps cell death of neurons not directly injured by axotomy.

Several mechanisms could account for the capacity of the approximately 70% of the normal complement of DRG neurons in adult operates and 25% of the normal number of DRG neurons in neonates to mediate SPIR recovery. 1. DRG neurons that synthesize SP might survive peripheral axotomy better than other populations. It seems unlikely, however, that SPIR-containing neurons were preferentially spared. SPIR has been found in 20% of the neurons in the DRG (Hökfelt et al., '76; Dalsgaard et al., '82; Panula et al., '83; Henken et al., '88) and is localized in neurons of the small to medium-size classes (Price, '85). We do not see any specific size of DRG neurons spared following either adult or neonatal sciatic nerve section, although the mean cell size in the L5 DRG of neonatally lesioned animals is significantly reduced. 2. Additional DRG neurons that can synthesize SP might be born postnatally. It has been proposed that neurogenesis occurs in the DRG throughout the life of the animal (Devor and Govrin-Lippmann, '85; Devor et al., '85) and if this is the case then among these new cells might be some that can synthesize SP. Our cell count data does not support a postnatal increase in DRG neurons. A linear regression analysis comparing L5 DRG cell numbers to the age of the animal using the unoperated left L5 DRG of neonatal operates (Table 3) shows the regression coefficient to be −0.3981. This result demonstrates that there is no postnatal increase and may in fact indicate a slight decrease in total cell number postnatally. Others have also failed to find evidence of postnatal neurogenesis using tritiated thymidine labeling experiments (Lawson et al., '74). 3. DRG neurons previously incapable of synthesizing SP might change their phenotype in response to axotomy and produce SP. Sciatic nerve section in adult rats has been shown to increase levels of vasoactive intestinal polypeptide (VIP) in DRG neurons that survived axotomy, even if VIP immunoreactivity was undetectable in DRG on the unoperated, control side (McGregor et al., '84; Shehab and Atkinson, '86; Shehab et al., '86). This is an indication that, at least in the adult DRG, neurons can increase or start up de novo synthesis of a substance not normally produced. 4. Numbers of SP-containing DRG neurons might not change, but local sprouting of surviving axotomized DRG neurons might increase their number of intraspinal SP-containing terminals. Counts of dorsal root axons have provided evidence for sprouting by newborn DRG neurons that survive forelimb amputation (Heath et al., '86). This process of reactive reinnervation of the partially deafferented dorsal horn would have to favor SP-containing fibers in order to explain the increased SPIR in the dorsal horn of chronic operates. 5. Axotomized DRG neurons might increase their SP synthesis, rate of axonal transport, and/or the turnover rate of SP in intraspinal terminals might be decreased. Mechanisms 3, 4, and 5 are consistent with our results and could account for the restoration of SPIR observed in both adult and neonatal operates.

Synthesis of SP by DRG neurons depends on the availability of NGF throughout the life of the neuron (Goedert et al., '81). The administration of NGF increases SP concentrations in newborn (Otten et al., '80; Kessler and Black, '80; Goedert et al., '81; Otten and Lorez, '83) and adult (Goedert et al., '81) DRG, whereas NGF antiserum decreases SP content at both stages (Ross et al., '81; Schwartz et al., '82). In addition, applying NGF locally to the proximal stump of the cut sciatic nerve prevents the expected decrease in SP levels within the DRG and spinal cord (Fitzgerald et al., '85). In the intact peripheral nerve, most NGF synthesis occurs in the target, and NGF reaches the neuronal cell body by retrograde axonal transport (Korsching and Thoenen, '83b); very little is synthesized along the course of the nerve (Korsching and Thoenen, '83a; Bandtlow et al., '87). After axotomy, NGF synthesis increases in the neuroma and in the immediately proximal stump of the nerve (Heumann et al., '87), where the increased synthesis of NGF by nonneuronal cells is thought to be stimulated by interleukin-1 released by invading macrophages (Heumann, '87; Lindholm et al., '87). Our observation that SPIR recovers in neurons that are prevented from regenerating implies that this locally synthesized NGF is capable of mediating recovery of SP. More robust recovery of SPIR in the spinal cord of neonates than in adults may be due to greater sensitivity to or higher levels of NGF (Bandtlow et al., '87) in the axotomized newborn peripheral nerve. Amounts of NGF-mRNA in the normal sciatic nerve of newborns have been reported to be comparable to those found in injured adult nerves (Heumann, '87). If still higher levels of NGF synthesis are stimulated by axotomy of the newborn sciatic nerve, perhaps more NGF is available per DRG neuron since fewer survive. These elevated levels of NGF-mRNA present after injury would cause increased synthesis of NGF, which would stimulate greater production of SP by surviving DRG neurons. This may provide the molecular basis for the robust recovery of SP that we have observed.

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In conducting research using animals, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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APPENDIX 21

The Biochemical Pathology of Astrocytes, pages 107-122
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ASTROGLIOSIS AND REGENERATION: NEW PERSPECTIVES TO AN OLD HYPOTHESIS

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INTRODUCTION

Reactive gliosis stands as one of the cardinal histopathological features of central nervous system (CNS) injury. Over the years this cellular response has taken on special significance in that the findings of many early investigations suggested that glial scars could impose an impediment to regeneration (for review see Reier et al., 1983). Thus, a fundamental hypothesis emerged concerning regenerative failure in the CNS, viz., that astrocytes prevent regeneration by establishing a physical barrier to growing axons. Consequently, considerable attention has been given to various cellular dynamics (e.g., proliferation, hypertrophy) underlying glial scar formation and the effects of astrocytes on axonal outgrowth (e.g., Eng et al., 1986).

The present chapter presents a selective overview of some current concepts related to the subject of gliosis and regeneration. Special emphasis is given to some of the more recent evidence related to the function of glial cells in the injured brain and spinal cord as seen in recent neural tissue transplantation studies. It should be noted that for the purposes of this review only a limited bibliography is cited; however, a more complete list of references and discussion is provided in two recent reviews of this topic (Reier, 1986 and Reier and Houle, 1988).

DO ASTROCYTES IMPEDE AXONAL OUTGROWTH?

The view that astrocytes, alone or with infiltrating connective tissue elements, establish barriers to regenerating axons stems largely from the early histological studies of the injured CNS wherein the growth of axons seemed to be arrested as they approached regions of dense glio-fibrotic scarring. More recently, however, the barrier concept has become less appealing as a mechanism whereby the astrocytic scar influences axonal elongation. This hypothesis has also been challenged on the basis that gliosis does not appear to be a primary determinant of the failure of regeneration as an absence of axonal regrowth has been frequently seen even where no glial reaction was apparent (Gilson and Stensaas, 1974; Guth et al., 1981). Ultrastructural studies of the injured spinal cord have also yielded observations inconsistent with the barrier concept (e.g., Matthews et al., 1979).

Despite these and other apparent exceptions (see Section VI in Reier, 1986) to the glial scar hypothesis, considerable evidence has been generated from several experimental models showing that mature astrocytes, whether reactive or non-reactive, can be prejudicial to the overall

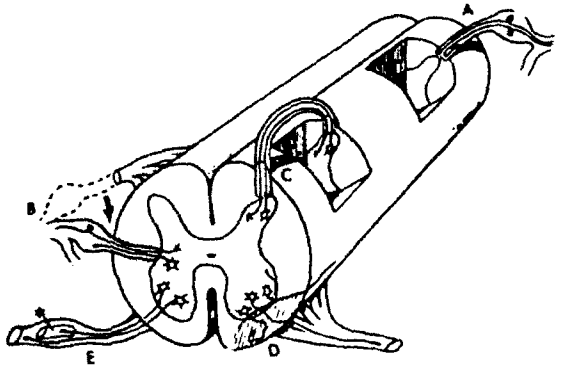

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Figure 1. This diagram summarizes various *in vivo* experimental models that have been used to study the effects of astrocytes on axonal outgrowth. See text for details and Reier, 1986 and Reier and Houle, 1988 for additional description of these models and pertinent references. Reprinted with permission from Reier and Houle, 1988.

success of a regenerative effort. As depicted in Figure 1, such studies have included analyses of regeneration: (i) at the dorsal root entry zone (DREZ, "A"), (ii) following insertion of the proximal stump of an injured peripheral nerve into the CNS (e.g., ectopic transposition of a dorsal root, "B"), (iii) after grafting of peripheral nervous system (PNS) tissue into the CNS ("C"), and (iv) following placement of CNS tissue between the stumps of a damaged peripheral nerve ("E").

In the case of the DREZ (e.g., Stensaas et al., 1986), it is known that regenerating primary afferent axons exhibit a robust growth capacity in the peripheral portion of the dorsal root. The majority of these fibers, however, are unable to advance further towards the CNS once they reach the PNS-CNS transition region where a dome or fringe of astrocytic processes is present. Interestingly, a substantial scar need not be established to impede axonal outgrowth (e.g., Carlstedt, 1985). That this cessation of regeneration may not just be due to some inherent metabolic limitation on the part of the dorsal root ganglion cell has been indicated by other studies showing that few, if any, motoneuron axons are able to traverse this region after ventral root-dorsal root anastomosis (e.g., Carlstedt,1983).

That the cellular microenvironment at the PNS-CNS transition zone of the DREZ influences regeneration has been further supported by recent transplantation studies. In these experiments, transected dorsal roots were juxtaposed to grafts of embryonic spinal cord tissue placed into lesions of the adult rat spinal cord (Tessler et al., 1987). As seen in Figure 2, injured, mature primary afferent fibers exhibit a rather dramatic innervation of fetal CNS tissue under these conditions. In another investigation, Carlstedt et al. (1986) observed an ingrowth of spinal sensory axons with similar histological techniques after crushing dorsal root fibers in the neonatal rat. This capacity to regenerate past the DREZ, however, was limited to the first week of postnatal life. Some change must therefore occur at the DREZ after this time which renders the microenvironment less favorable to axonal elongation. These results are consistent with other studies showing that the ability of immature glia to be compatible with axonal elongation in other parts of the CNS is also limited to early postnatal life in the rodent (Grafe and Shoenfeld, 1982, Sijbesma and Leonard, 1986; Smith et al., 1986).

Figure 2. Insertion of a transected dorsal root (DR) into an intraspinal graft of fetal spinal cord tissue (FSC). Growth of primary afferent fibers past the DR-FSC junction (arrowheads) is demonstrated by immunocytochemical staining with antibody to Calcitonin Gene-Related Peptide. H = host cord. X 95.

The findings from studies of the DREZ have also been mirrored to a considerable extent by experiments in which the regenerating proximal stump of a peripheral nerve has been inserted into the substance of the CNS (Fig. 1, B). For example, Carlstedt (1985) reported that following the introduction of dorsal roots into ectopic regions of the spinal cord, sensory axons were still unable to advance centrally even though only a minimal astroglial matrix developed at the insertion site.

A compelling demonstration of the importance of the glial microenvironment has also derived from studies of PNS grafts to the CNS (reviewed in Aguayo, 1985). These studies have shown that neurons intrinsic to the CNS can extend their axons for long distances in the presence of PNS tissue. Upon approaching the opposite end of these grafts, however, the CNS axons, as seen in other studies (e.g., Fig. B), fail to grow for more than 1-2 mm. To some extent, this may be related to the development of an encapsulating glial scar at the graft-CNS junction (Fig. 3a; see also, Reier, 1985 for other references).

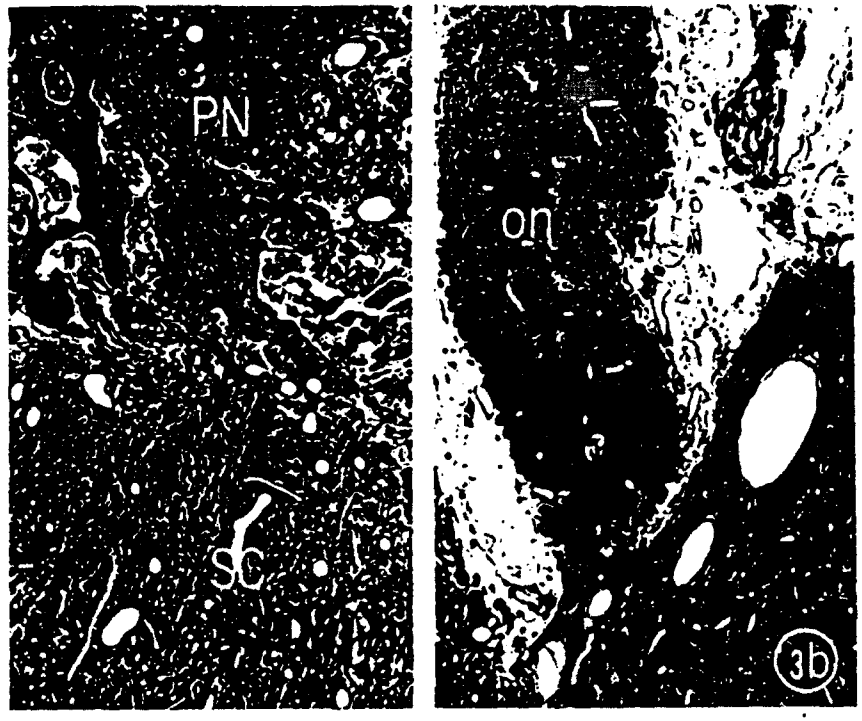

Figure 3. (a) A plastic thick section showing the insertion site of a peripheral nerve autograft (PN) into the spinal cord (SC). Arrowheads indicate some scarring around the end of the nerve implant. A capsule of dense astrocytic scar tissue is also seen along the opposing surface of the spinal cord (arrows). Note the presence of numerous myelinated axons in the peripheral nerve graft. X190. (b) A segment of optic nerve glial scar tissue (on) inserted into the brainstem of an adult rat. Despite close approximation (as with PNS grafts) with the CNS neuropil, no evidence of axonal ingrowth is indicated. X190.

As could be predicted from the studies of the experimental models described thus far, grafts of CNS tissue (i.e. optic nerve) to an injured peripheral nerve (Fig. 1, "E") are not readily penetrated by regenerating axons (Aguayo et

., 1978, Weinberg and Spencer, 1979; Reier et al., 1983; derson and Turmaine, 1986; Hall and Kent, 1987) even ough a partition of astrocytic processes or basal lamina n be absent at the graft-sciatic nerve interface (Hall and nt, 1987). Likewise, axonal elongation does not occur in- optic nerve glial scar segments (see Reier et al., 1983 d Reier, 1986 for additional details concerning production the scar implant) after being grafted to various regions the brain (Fig. 3b; Reier, unpublished observations).

The results obtained with PNS-to-CNS and CNS-to-PNS rafts have been corroborated by recent in vitro studies. Schwab and Thoenen (1985) and Carbonetto et al. (1987) ave observed that in co-cultures consisting of peripheral eurons along with PNS and CNS tissue elements, neuritic utgrowth occurred preferentially in the presence of a PNS ubstratum, whereas the CNS microenvironment was virtually voided. This attraction of axons to peripheral nerve issue may be even stronger than revealed by these in itro studies. Some in vivo experiments have shown hat the same glial microenvironment which impedes the ad- ance of axons into the CNS may not, at least in some cases, inder the extension of axons when PNS tissue is present at he other end of the glial scar (Fig. 1, "B", "C" and "D", ee also Risling et al., 1983).

In addition to the in vivo and in vitro evidence mentioned, an indication that scar tissue (glial and mesodermal tissue) may compromise axonal outgrowth has also evolved from other transplantation studies. For example, when fetal CNS tissue is grafted into a cavity made in the adult spinal cord, varying degrees of host-graft fusion are achieved (Reier et al., 1986). Some neuritic elongation occurs where a confluent neuropil is established between host and graft (Fig. 4a); however, no axonal outgrowth either to or from the transplant is suggested when a dense cellular partition is formed at this interface or when a glial capsule develops along the surface of the grafts (Fig. 4b). The absence of fiber outgrowth from transplants under such conditions is especially significant given that the immature donor neurons, despite possessing an optimal growth potential, still seems unable in most cases to extend their cytoplasmic processes into a dense astrocytic matrix.

While these various lines of evidence point to the astrocyte as having a singular adverse effect on axonal

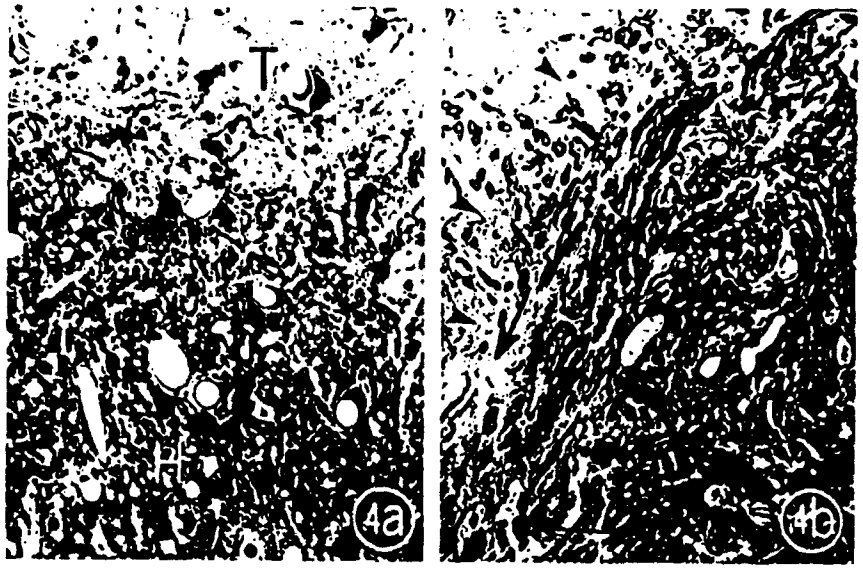

Figure 4. Two micron sections showing (a) a confluent neuropil between host spinal cord (H) and a fetal spinal cord transplant (T) and (b) the formation of a dense astrocytic capsule (arrowheads) over the surface of a fetal spinal cord transplant which did not become integrated with the host spinal cord. Note the infiltration of connective tissue elements (arrows) between host and graft. Also, note the paucity of axons in the astroglial matrix at the edge of the transplant. X200.

elongation, Schwab et al. (1987) have concluded from their recent studies that mature oligodendroglia and myelin-related proteins can establish non-permissive substrata for regenerating neurites in culture. This suggestion serves as an important reminder that glial scars in many instances are not comprised solely of reactive astrocytes and that the effect of such scar tissue on axonal growth may thus be due to similar actions exerted by other cell types either alone or in combination with the astrocyte. Nevertheless, robust axonal elongation through the matrix of a glial scar, consisting predominantly of astrocytic elements, is more the exception rather than the rule. This argues that the mature astrocyte does have an adverse influence on fiber outgrowth or is simply unable to support it. Therefore, the issue is not whether astrogliosis can prejudice regeneration, but

mechanism(s) do these cells influence axonal regrowth and sprouting.

HOW DO ASTROCYTES EXERT AN INFLUENCE ON GROWING AXONS?

Although examination of static histological preparations often suggests that glial scar tissue acts as a physical obstacle to growing neuritic processes, other mechanisms could yield the same picture. Thus, several alternatives to the glial barrier hypothesis have been proposed (reviewed in Reier and Houle, 1988).

In general, most of the newer hypotheses assert on one hand that reactive glia produce growth-inhibiting factors and/or cell surface constituents. On the other hand, the failure of axons to extend in an astroglial microenvironment may be due to the inability of the mature astrocyte to synthesize the appropriate neurite-promoting molecules. For example, extracellular matrix (ECM) constituents believed to be positive effectors of axonal elongation in the PNS are absent in CNS tissue. In addition to CNS tissue being deficient in laminin, fibronectin, and heparin sulfate proteoglycan (HeSPG) (Carbonetto et al., 1987), recent evidence points to a laminin-HeSPG complex as being another physiological substratum for axonal outgrowth in the PNS which is absent in the CNS (Chiu et al., 1986). As pointed out elsewhere in this volume (see Chapter by Bignami et al), the possibility cannot be overlooked that an ECM is present in the CNS which is inhibitory to axonal outgrowth.

Other studies have suggested that growth-associated triggering factors (Hadani et al., 1984; Schwartz et al., 1985) present in regenerating optic nerves of fish and in the developing optic nerves of newborn rabbits are also lacking in injured adult mammalian CNS tissue. While it appears that such substances are being produced by non-neuronal cells in the fish and newborn rabbit, their biosynthesis by astrocytes remains to be shown.

Proteases (Kalderon, 1987a, b) and protease inhibitors (Geunther et al., 1985; Monard, 1985) are also thought to be related to axonal extension, and an imbalance between these substances may account for the limited outgrowth in most astroglial matrices (see also discussion by Stensaas et al., 1987). Kalderon (1987a, b), for instance, has reported that plasminogen activator (PA) activity is low in mature astroglial populations but high in the immature astrocyte. In contrast, protease inhibitory activity is a major featu of mature and reactive astrocytes. Addition of the inhibi to injured peripheral nerves can impair regeneration.

IS THE EFFECT OF ASTROCYTES ENTIRELY ADVERSE TO CNS REGENERATION AND PLASTICITY?

The inability of mature astrocytes to foster axona growth has also been frequently attributed to this cell be ing unable to synthesize necessary trophic factors. This view has been partly challenged, however, by the recent in vitro studies of Schwab and Thoenen (1985) in which axons failed to invade optic nerve tissue despite the presence o NGF in the culture medium. Furthermore, as discussed in recent reviews (Cotman and Nieto-Sampedro, 1984; Manthorpe al., 1986; Lindsay, 1986), other lines of evidence suggest that astrocytes produce various neuronotrophic factor (NTFs). For example, a high level of NTF activity has bee detected in fluids which accumulate at sites of CNS injury Accordingly, such fluids have the capacity to promote the survival of various neuronal types in culture, as well as certain transplants of fetal CNS tissue. Since the most e evated trophic titers were detected within tissue lining t walls of lesion cavities, it has been proposed that such activity might be associated with glial cells as they reestablish a glial limiting membrane along the injured surfaces of the brain. The time-course of this trophic activity induction also paralleled the onset of gliosis.

While suggesting that astrocytes are capable of producing NTFs, limited regeneration in vivo may still be due to insufficient synthesis of such factors. What is produced may only be adequate for inducing short distance sprouting of fibers. Furthermore, it is possible that glial-derived factors in the adult CNS may exert a more profound regulatory effect in some cases upon reactive synaptogenesis. This type of action may in turn lead to the cessation of axonal elongation. It is worth noting in this context that several workers have proposed that astrocytes can hinder axonal elongation by inducing the formation of presynaptic terminals (see Reier and Houle, 1988 for reference and further discussion).

Although more has to be learned concerning the astrocyte's ability to produce trophic substances in vivo, the available evidence serves to underscore that these cells may have a greater overall beneficial effect on injured neurons than previously recognized. In this regard, it is possible that astrocytes may actually establish a dichotomous microenvironment which is so favorable to the neuron that it ultimately discourages any further growth.

EMBRYONIC ASTROCYTES AND THEIR INFLUENCE ON THE INJURED MATURE CNS

Over the last several years, there has been a rapidly growing interest in neural tissue transplantation. Aside from this approach showing that it is possible to replace various neuronal populations with grafts of embryonic CNS tissue, some studies have also demonstrated that it is feasible to transplant enriched populations of astroglial cells derived from either tissue culture (Kesslak et al., 1986) or by harvesting young astrocytes which attach to artificial membranes placed into brain lesions (Silver and Ogawa, 1983; Smith et al., 1986). Such investigations have cast light on the potential of immature nonneuronal elements for modulating glial and other cellular responses to trauma in the CNS.

In one set of experiments, Smith et al. (1986) have observed that implants of immature glia, attached to a Millipore strip and introduced into cortical lesions, reduced gliosis, bleeding, and secondary necrosis, which generally occurred in the absence of such grafts. These findings are intriguing in view of other reports, discussed earlier, showing that immature astrocytes exhibit a high level of PA activity. Furthermore, Hadani et al. (1984) have shown that substances in neonatal rabbit optic nerve - conditioned medium may lead to a stimulation of regeneration in the mature rabbit visual system. This may occur through activation or modulation of the the biosynthetic activities of scar-forming astrocytes (Schwartz et al., In Press).

Recent findings related to the intraspinal transplantation of fetal spinal cord tissue into acute and chronic lesions of the adult rat spinal cord (Reier et al., 1986; Bregman and Reier, In Press) are also consistent with the apparent ability of immature glia to reverse some of the pathology caused by CNS injury. In the former case, transplantation performed immediately after injury results in numerous sites of host-graft fusion without any intervening gliosis (Fig. 4a). Some areas of intimate host-donor tissue integration can also be observed following transplantation into lesion cavities made 2-7 weeks prior to grafting (Fig. 5a). By two weeks in this case the original wound is lined by a continuous multilayered astrocytic capsule. While fusion of the graft with the host spinal cord is never complete in either the acute or chronic lesion preparation (Fig. 5b), the

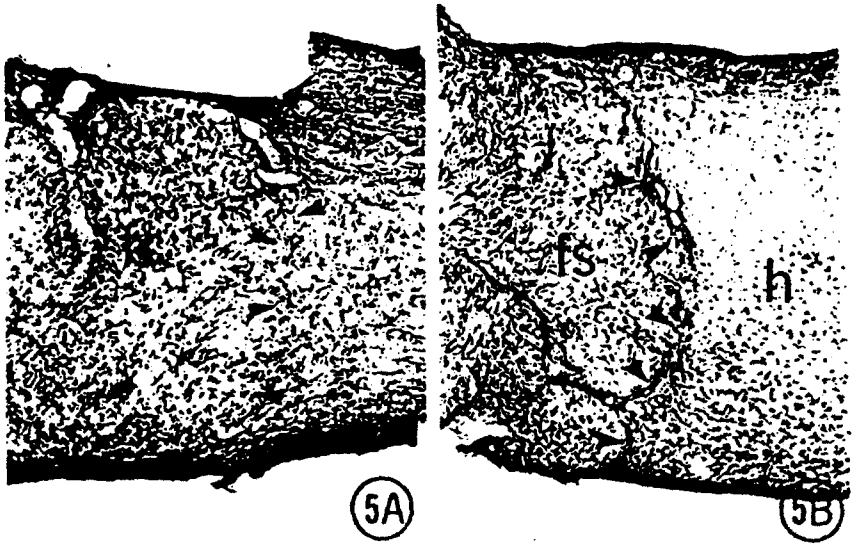

Figure 5. Transplantation into a chronic (7-week) lesion of the adult spinal cord. (a) A parasagittal section, stained with anti-GFAP, showing the rostral interface (arrowheads) between a fetal spinal cord graft (fc) and the host spinal cord 4 months after transplantation. Note the absence of a defined glial partition at the host-donor junction (arrowheads). (b) Regression of the glial scar formed at the time of transplantation is variable, however, as seen in this more distant anti-GFAP-stained section of the same specimen illustrated in "a". Arrowheads indicate the presence of some glial scar tissue at the host (h) - graft (fs) interface. X200.

fact that a continuous neuropil can be established in both instances suggests that some property of immature CNS tis-

sue, possibly related to the population of young astrocytes, may be capable of inhibiting gliosis (acute injury recipients) or of modifying an existing glial scar (chronic lesion hosts). The fact that only partial suppression or regression of the scar was achieved in both types of lesion preparations may reflect a limited developmental period during which an effect can be exerted by the fetal graft (Smith et al., 1986).

CONCLUSION

Over the last decade, there has been a progressive shift toward a more optimistic scientific attitude concerning the feasibility of CNS repair. It is now known that many neurons intrinsic to the adult CNS have the potential to extend their axons over long distances provided that the appropriate tissue microenvironment is available. While the astrocyte is not a primary determinant of the outcome of any spontaneous regenerative effort, many lines of evidence indicate that this cell still plays a vital role, no matter how far removed, in governing the general success of regeneration by establishing a cellular matrix which in some way discourages axonal elongation. Somewhat paradoxically, however, this cell also plays a very major part in a natural healing process after CNS injury and may produce substances essential to the general well-being of the neuron, to the preservation of synaptic circuitries, as well as to the expression of plasticity in the damaged CNS.

Further progress in relation to regeneration and neural plasticity clearly demands a better understanding of the cellular biology of the astrocyte and its metabolic and synthetic properties in vivo and in vitro. It is encouraging that some advances have already been made in this direction leading to new concepts about the astrocyte's role in the injured CNS. Such fresh perspectives can be instrumental in ultimately defining future therapeutic approaches. For example, an ideal strategy might be one designed to modulate the astrocyte's response to injury so as to gain from its trophic effect while at the same time tempering its scarring response. Studies showing that some glial responses in the mature non-regenerating CNS can be altered by fetal grafts, immature astrocytes, or chemical properties of developing mammalian and regenerating nonmammalian CNS tissue suggest that research may be at the threshold of exciting breakthroughs in our understanding of how to control the astrocyte's response to CNS injury.

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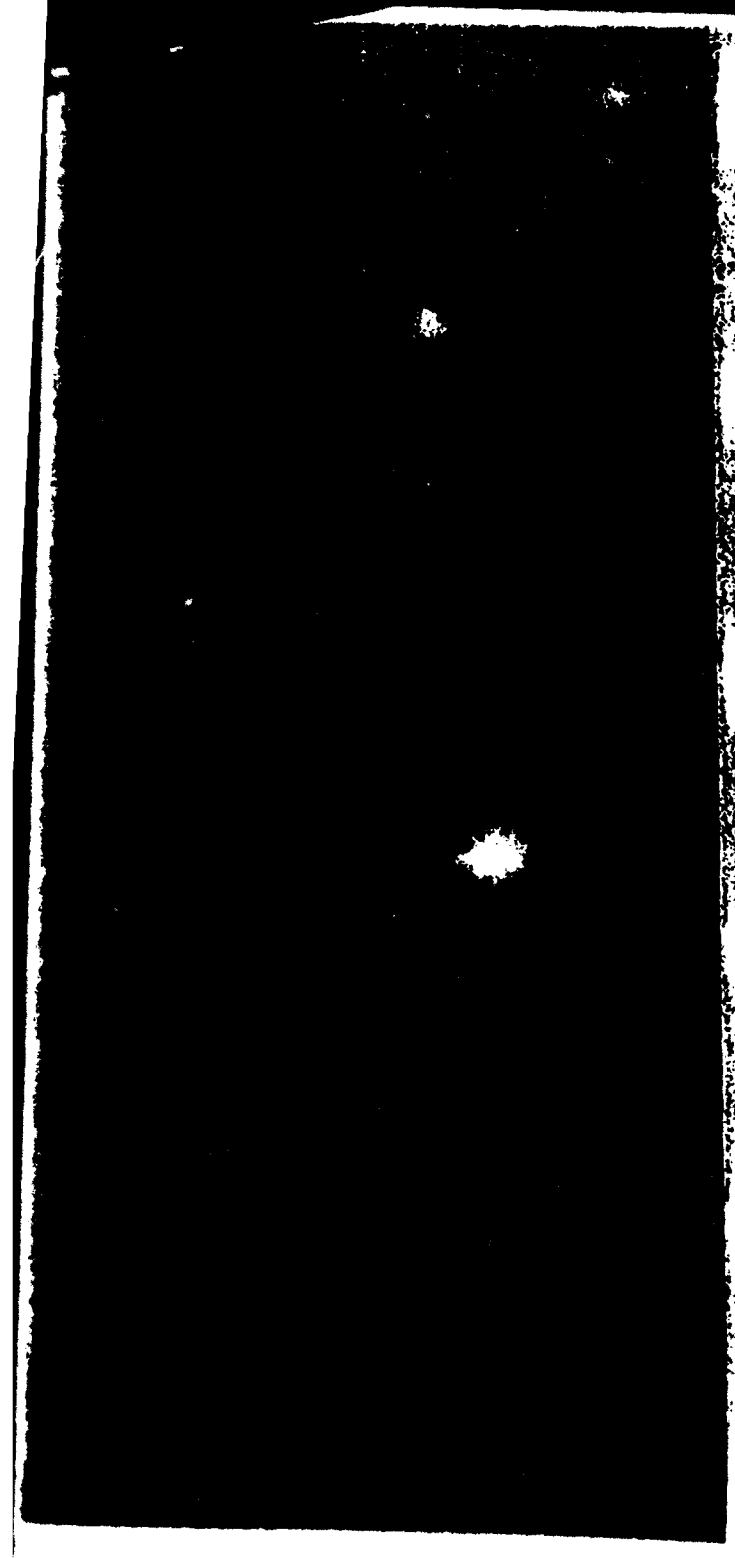

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